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BLOOD

The Journal of The American Society of Hematology

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The Proven Con

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Wellcovorin Tablets are dramatically improving the way patients are rescued. Often discharged from the hospital earlier, more patients are completing their therapy on an outpatient basis and returning to a more normal, comfortable lifestyle sooner.

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[1] Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.

Reducing the Cost of Rescue

Wellcovorin Tablets have reduced the cost of leucovorin rescue significantly. Before Wellcovorin Tablets, the cost of leucovorin increased rapidly and consistently every year. Now, patients pay less for Wellcovorin Tablets, and often realize considerable savings from a reduction in hospital stays and clinic visits.

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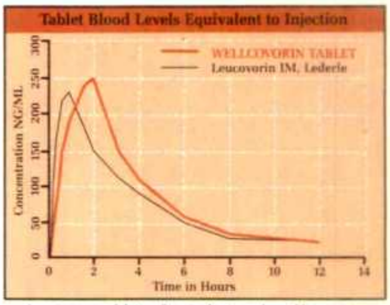
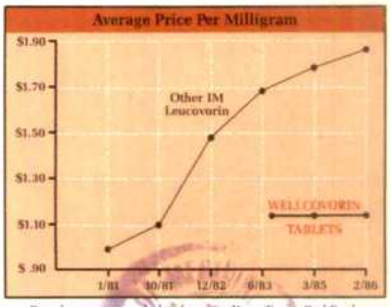


Average serum folates after single 15 mg dose of leucovorin administered either IM or orally. Adapted from Blum, et al.

Blum MR, Guaspari A, Liao SHT, et al: Bioavailability and pharmacokinetics of oral and parenteral leucovorin. 33rd National Meeting Pharm. Sci. San Diego, California. 1982. 12(2):175.



Based on average wholesale price. *Drug Topics Red Book*. Medical Economics Company.

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Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer, 41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al:* High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med, 68*:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro. Br J Haematol, 40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al:* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis. 135*(3): 493-496, 1977.

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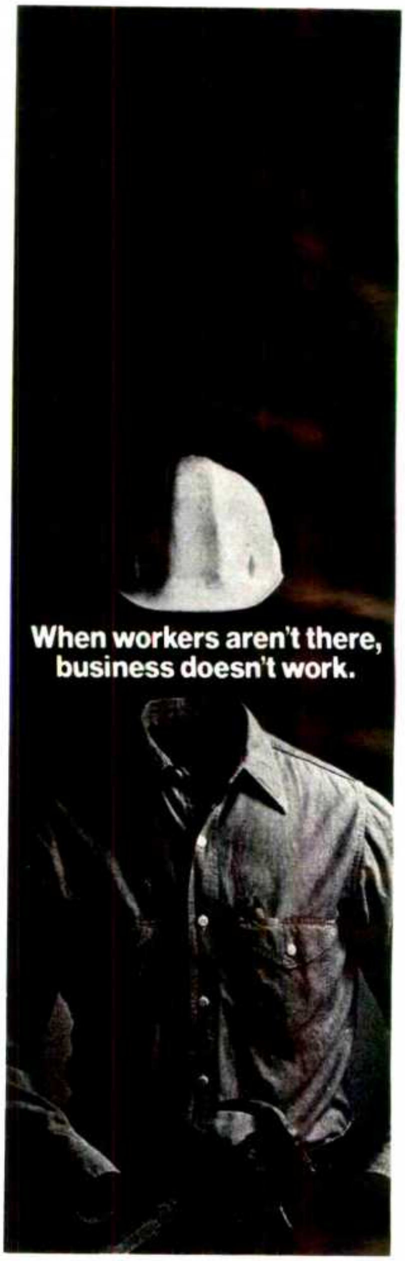
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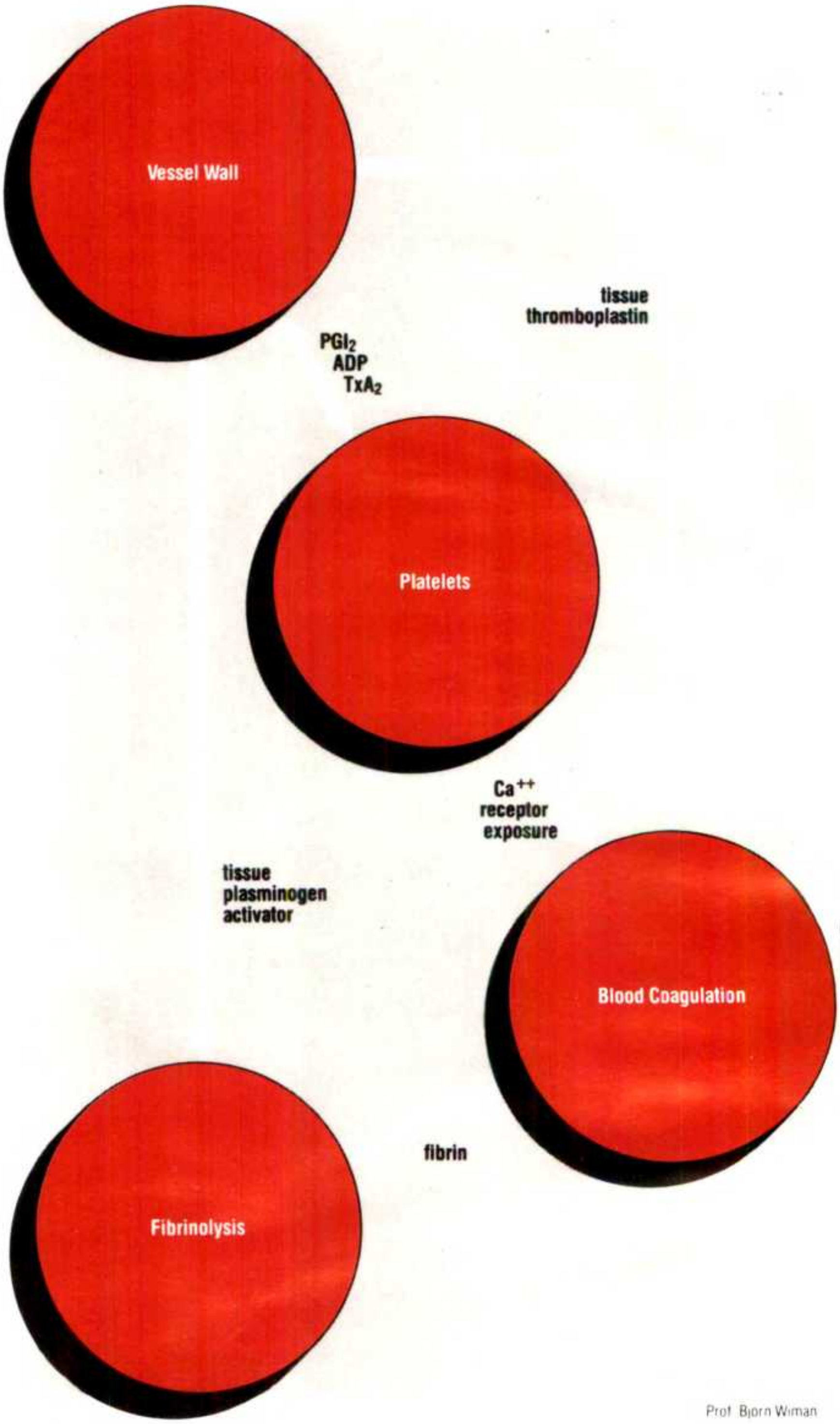

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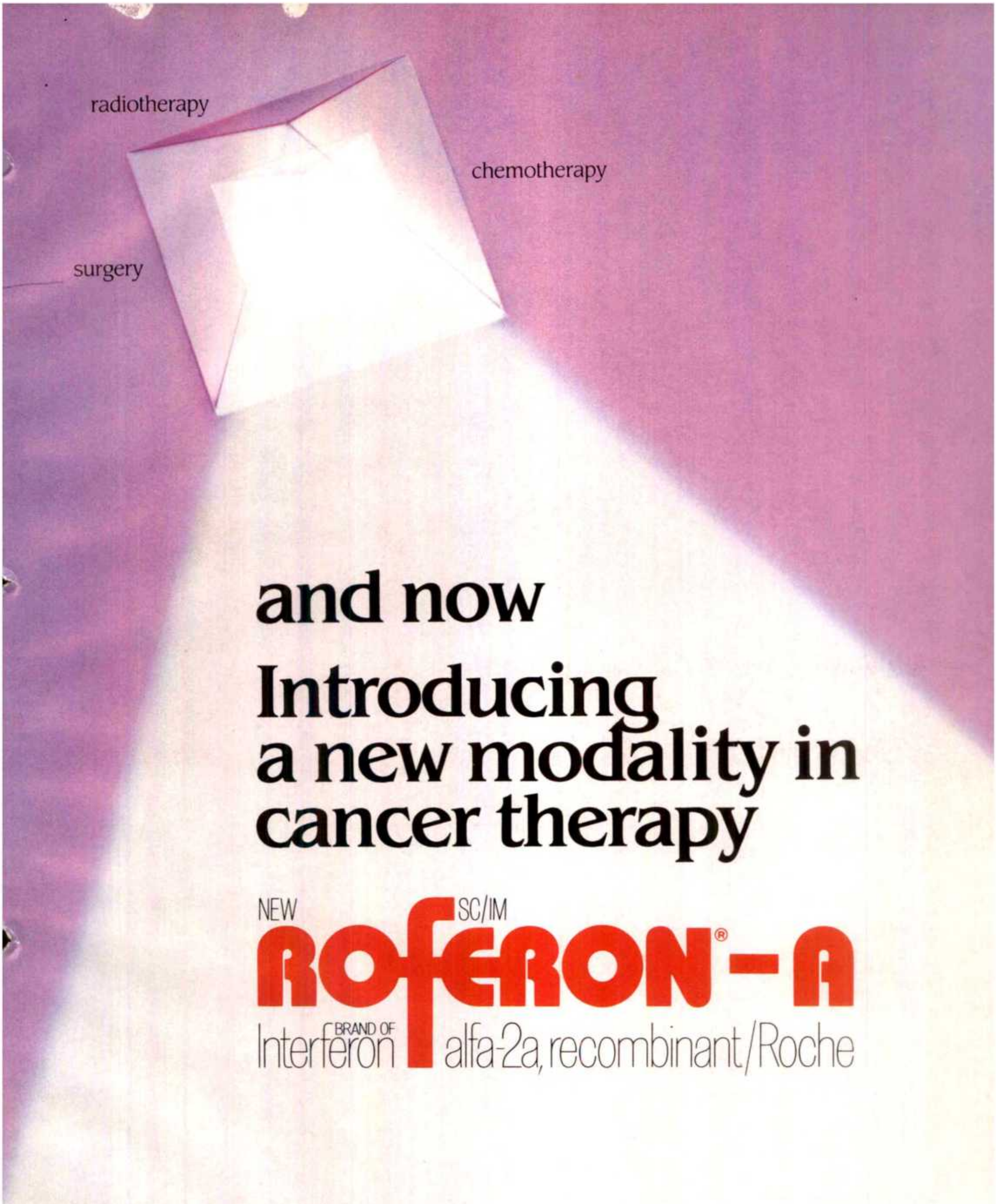

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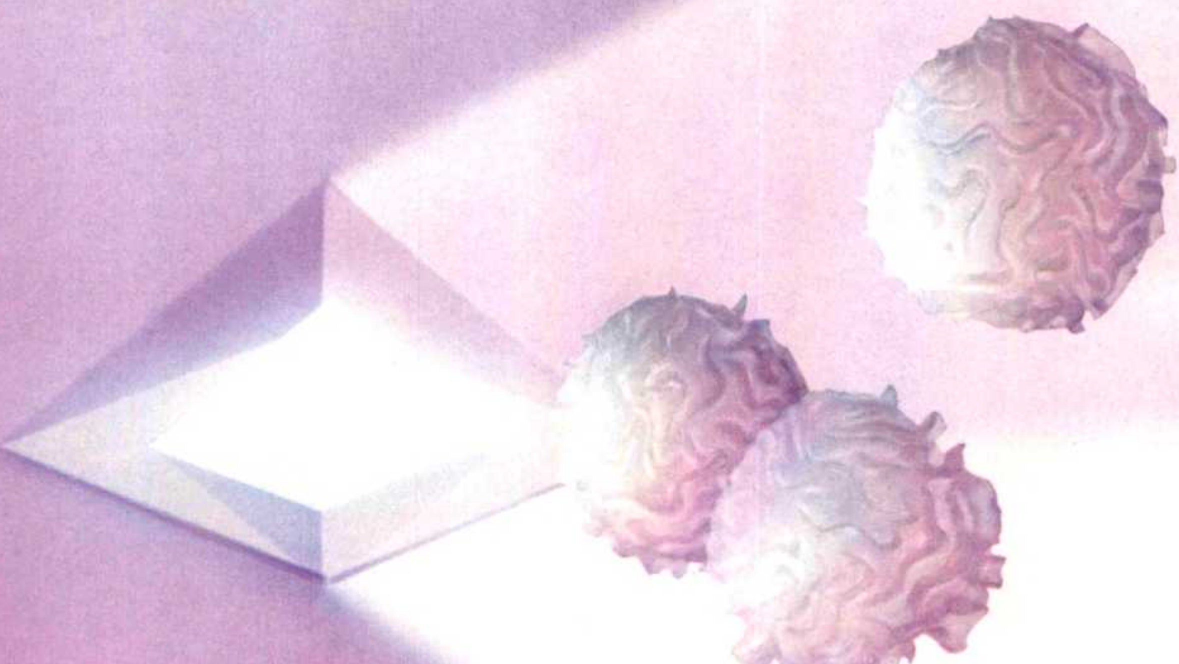

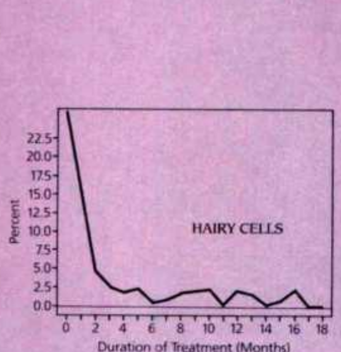

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Hemoglobin	> 12 Gm/dL	> 12 Gm/dL	Improvement
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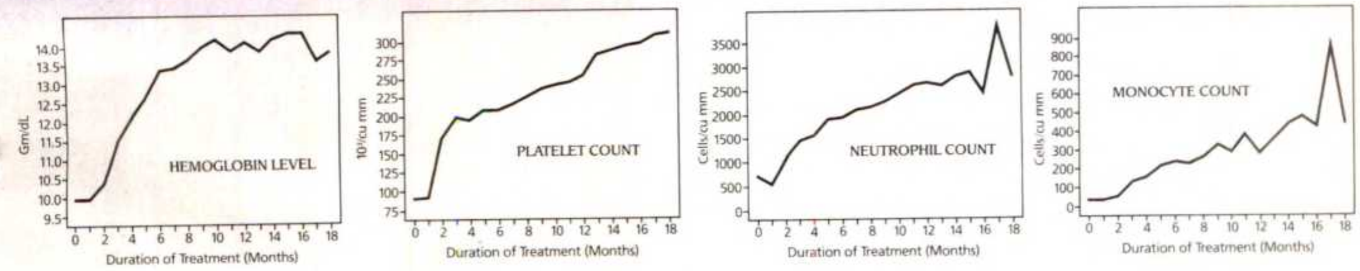
Platelet Count	> 100,000/cu mm	> 100,000/cu mm	Improvement

Normalization of blood counts*



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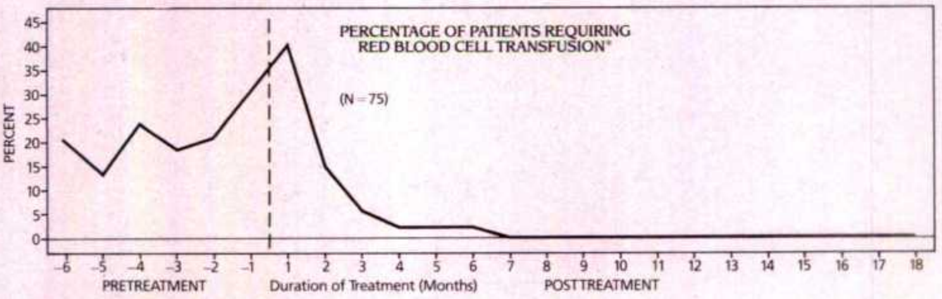
[*] Multicenter Study, Data on file, Hoffmann-La Roche Inc., Nutley, NJ.

[†] Of the 75, 57 had prior splenectomy and 18 had not; many were pancytopenic and transfusion-dependent; 96 patients were enrolled in study and all were evaluated for safety; 21 patients were not evaluable; 16 were not on drug long enough for efficacy evaluation at time of data analysis, 2 were removed for administrative reasons, 3 for preexisting intercurrent illness.

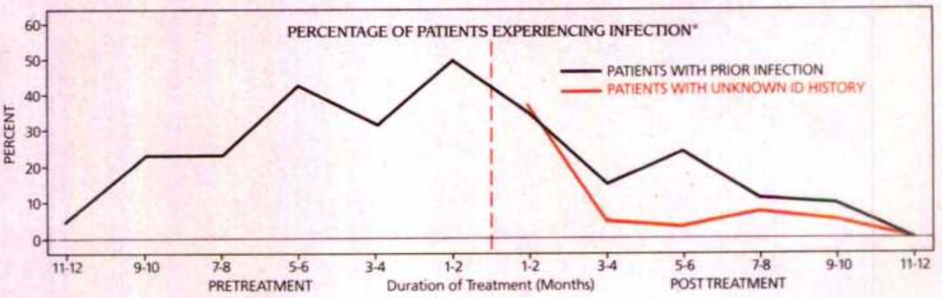
A new modality...
ROCHE Leading the way in biotherapeutics

a better quality of life

From transfusion dependency to independent ambulatory treatment



From frequent life-threatening opportunistic infections to virtually none



*Multicenter Study, Data on file, Hoffmann-La Roche Inc., Nutley, NJ.

From frequent hospital visits to self-injection at home

After an initial period of hospital or clinic visits for drug administration, self-injection at home or administration by a family member makes it possible for many patients to lead a more normal life.

From the serious, difficult-to-manage side effects of chemotherapy to the more manageable side effects of biotherapy

The common side effects of ROFERON-A are rarely as debilitating as some of the serious side effects associated with standard chemotherapy and radiotherapy. (See side effects listed on following pages and in complete product information on last pages of this advertisement.)

From weight loss to weight stabilization

In a multicenter study, 86% of patients on ROFERON-A experienced no change in, or gained up to 10% of, their total body weight.

NEW SC/IM

ROFERON®-A

BRAND OF
Interferon alfa-2a, recombinant/Roche

Before prescribing, please see complete product information on last pages of this advertisement.

Guidelines to treatment

Patient selection

ROFERON-A (Interferon alfa-2a, recombinant/Roche) is indicated for symptomatic adult patients. It should be used with caution in patients with preexisting cardiac disease, renal and hepatic disease, myelosuppression, seizure disorders and/or compromised central nervous system function.

Information for patients

Patients should be cautioned not to change brands of Interferon without medical consultation, as a change in dosage may result. They should also be informed regarding the potential benefits and risks attendant to the use of ROFERON-A. If home use is judged desirable, instructions on appropriate use and self-injection should be given.

Patients should be well hydrated, especially during the initial stages of treatment.

Dosage and administration

Induction:	**3 million IU SC or IM once daily for 6 months.**
Maintenance:	**3 million IU SC or IM 3 times a week.**
Dosage Reduction:	**If severe reactions occur, reduce dosage by 50% or withhold individual doses.**

How supplied

Ready-to-use Injectable Solution: Unit-dose vials containing 3 million IU (3 million IU/mL); multidose vials containing 18 million IU (3 million IU/0.5 mL).
No mixing or reconstitution necessary.
Must be refrigerated at temperatures of 36° to 46°F (2° to 8°C). At no time should ROFERON-A (Interferon alfa-2a, recombinant/Roche) Injectable Solution remain outside of refrigeration for more than 24 hours. Be certain all users, including physicians, nurses and patients are aware of this important precaution. Do *not* freeze or shake.

Directions for use

The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are significantly thrombocytopenic or at risk for bleeding.

Side effects: generally mild and manageable

Pretreatment

Routine history and physical examination. Determine baseline values: CBC, peripheral and bone marrow hairy cells, liver and renal function.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Flu-like symptoms

The most common initial reactions, fever, chills, myalgia, headache and fatigue, are usually self-limiting and can be ameliorated by acetaminophen.

Myelosuppressive effect

The myelosuppressive effect warrants careful monitoring for depression of blood cell

ROCHE Leading the way in biotherapeutics

counts during initial therapy and is usually alleviated by dose reduction or temporary discontinuation of drug.

Other common but generally mild side effects

These include anorexia, nausea, vomiting, diarrhea, dizziness, rash, change in taste, dry mouth or throat, dry skin or pruritus and weight loss. Persistent mild fatigue, a problem for many patients, is usually found to be less troublesome when ROFERON-A is administered *p.m.* or *h.s.*

Rarely reported but more serious side effects

These include, but are not limited to, central nervous system effects such as decreased mental status, depression, visual disturbances, sleep disturbances and nervousness, as well as such adverse cardiac events as hypertension, chest pain, arrhythmias and palpitations.

Patient monitoring

Monitor blood parameters, liver and renal function periodically (*e.g.*, monthly) to determine if patient is responding to and tolerating drug. Response is not generally observed for one to three months. Patients usually should be treated for approximately six months before determining whether to continue therapy.

Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.

Laboratory findings

No patient had to be removed from multicenter study due to clinical laboratory abnormality.

Laboratory abnormalities, observed frequently but generally not of clinical significance, include:

SGOT elevation

Common but not dose-limiting

Neutralizing antibodies

Found in four out of 51 (7.8%) patients in multicenter study evaluable for the development of antibodies; no clinical sequelae or adverse effect on response, duration of response or survival has been noted thus far.

NOTE: Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.

Contraindications

ROFERON-A is contraindicated in patients with known hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

Use in fertile women

Fertile women should use effective contraception while receiving ROFERON-A.

NEW SC/IM

ROFERON®-A

BRAND OF Interferon alfa-2a, recombinant/Roche

Before prescribing, please see complete product information on last pages of this advertisement.

NEW SC/IM

ROFERON®-A

BRAND OF
Interferon alfa-2a, recombinant/Roche

DESCRIPTION: Roferon®-A (Interferon alfa-2a, recombinant/Roche) is a sterile protein product for use by injection. Roferon-A is manufactured by recombinant DNA technology that employs a genetically engineered *E. coli* bacterium containing DNA that codes for the human protein. Interferon alfa-2a, recombinant/Roche is a highly purified protein containing 165 amino acids, and it has an approximate molecular weight of 19,000 daltons. The purification procedure includes affinity chromatography using a murine monoclonal antibody. Roferon-A is supplied as an injectable solution. The solution is colorless and contains Interferon alfa-2a, recombinant/Roche, phenol as a preservative, sodium chloride for isotonicity and human serum albumin as a stabilizer. Fermentation is carried out in a defined nutrient medium containing the antibiotic tetracycline hydrochloride, 5 mg/liter. However, the presence of the antibiotic is not detectable in the final product.

Each vial of Roferon-A contains 3 million or 18 million international units (IU) of Interferon alfa-2a, recombinant/Roche. The specific activity of Interferon alfa-2a, recombinant/Roche, is 2×10^8 IU/mg protein. The route of administration is subcutaneous or intramuscular.

CLINICAL PHARMACOLOGY: The mechanism by which Interferon alfa-2a, recombinant/Roche, or any other interferon, exerts antitumor activity is not clearly understood. However, it is believed that direct antiproliferative action against tumor cells and modulation of the host immune response play important roles in the antitumor activity.

The biological activities of Interferon alfa-2a, recombinant/Roche are species-restricted, *i.e.*, they are expressed in a very limited number of species other than humans. As a consequence, preclinical evaluation of Interferon alfa-2a, recombinant/Roche has involved *in vitro* experiments with human cells and some *in vivo* experiments.[1] Using human cells in culture, Interferon alfa-2a, recombinant/Roche has been shown to have antiproliferative and immunomodulatory activities that are very similar to those of the mixture of interferon alfa subtypes produced by human leukocytes. *In vivo*, Interferon alfa-2a, recombinant/Roche has been shown to inhibit the growth of several human tumors growing in immunocompromised (nude) mice.

Because of its species-restricted activity, it has not been possible to demonstrate antitumor activity in immunologically intact syngeneic tumor model systems, where effects on the host immune system would be observable. However, such antitumor activity has been repeatedly demonstrated with, for example, mouse interferon-alfa in transplantable mouse tumor systems. The clinical significance of these findings is unknown.

The metabolism of Interferon alfa-2a, recombinant/Roche is consistent with that of alfa interferons in general. Alfa interferons are totally filtered through the glomeruli and undergo rapid proteolytic degradation during tubular reabsorption, rendering a negligible reappearance of intact alfa interferon in the systemic circulation. Small amounts of radiolabeled Interferon alfa-2a, recombinant/Roche appear in the urine of isolated rat kidneys, suggesting near complete reabsorption of Interferon alfa-2a, recombinant/Roche catabolites. Liver metabolism and subsequent biliary excretion are considered minor pathways of elimination for alfa interferons.

The serum concentrations of Interferon alfa-2a, recombinant/Roche reflected a large intersubject variation in both healthy volunteers and patients with disseminated cancer.

In healthy people, Interferon alfa-2a, recombinant/Roche exhibited an elimination half-life of 3.7 to 8.5 hours (mean 5.1 hours), volume of distribution at steady-state of 0.223 to 0.748 L/kg (mean 0.400 L/kg) and a total body clearance of 2.14 to 3.62 mL/min/kg (mean 2.79 mL/min/kg) after a 36 million IU (2.2×10^8 pg) intravenous infusion. After intramuscular and subcutaneous administrations of 36 million IU, peak serum concentrations ranged from 1500 to 2580 pg/mL (mean 2020 pg/mL) at a mean time to peak of 3.8 hours and from 1250 to 2320 pg/mL (mean 1730 pg/mL) at a mean time to peak of 7.3 hours, respectively. The apparent fraction of the dose absorbed after intramuscular injection was greater than 80%.

The pharmacokinetics of Interferon alfa-2a, recombinant/Roche after single intramuscular doses to patients with disseminated cancer were similar to those found in healthy volunteers. Dose proportional increases in serum concentrations were observed after single doses up to 198 million IU. There were no changes in the distribution or elimination of Interferon alfa-2a, recombinant/Roche during twice daily (0.5 to 36 million IU), once daily (1 to 54 million IU), or three times weekly (1 to 136 million IU) dosing regimens up to 28 days of dosing. Multiple intramuscular doses of Interferon alfa-2a, recombinant/Roche resulted in an accumulation of 2 to 4 times the single dose serum concentrations. Pharmacokinetic information in patients with hairy cell leukemia is presently unknown.

The acute parenteral toxicity of Interferon alfa-2a, recombinant/Roche has been studied in mice, rats, rabbits and ferrets at doses up to 30 million IU/kg intravenously, and 500 million IU/kg intramuscularly. No treatment-related mortality was noted in any species given Interferon alfa-2a, recombinant/Roche by any of the routes of administration.

Effects on Hairy Cell Leukemia: During the first one to two months of treatment of patients with hairy cell leukemia, significant depression of hematopoiesis was likely to occur. Subsequently, there was improvement in circulating blood cell counts.

Of the 75 patients who were evaluable for efficacy following at least 16 weeks of therapy, 46 (61%) achieved complete or partial response. Twenty-one patients (28%) had a minor remission, eight (11%) remained stable, and none had worsening of disease. All patients who achieved either a complete or partial response had complete or partial normalization of all peripheral blood elements including hemoglobin level, white blood cell, neutrophil, monocyte and platelet counts with a concomitant decrease in peripheral blood and bone marrow hairy cells. Responding patients also exhibited a marked reduction in red blood cell and platelet transfusion requirements, a decrease in infectious episodes and improvement in performance status. The probability of survival for two years in patients receiving Roferon-A (94%) was statistically increased compared to a historical control group (75%).

ROFERON®-A (Interferon alfa-2a, recombinant/Roche)

INDICATIONS AND USAGE: Roferon-A is indicated for use in the treatment of hairy cell leukemia in people 18 years of age or older. Studies have shown that Roferon-A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and nonsplenectomized patients.[2,3]

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically (*e.g.*, monthly) during treatment to determine whether response to treatment has occurred. If a patient does not respond within six months, treatment should be discontinued. If a response to treatment does occur, treatment should be continued until no further improvement is observed and these laboratory parameters have been stable for about three months. It is not known whether continued treatment after that time is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS: Roferon-A is contraindicated in patients with known hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

WARNINGS: Roferon-A should be administered under the guidance of a qualified physician. (See DOSAGE AND ADMINISTRATION.) Appropriate management of the therapy and its complications is possible only when adequate diagnostic and treatment facilities are readily available.

Roferon-A should be used with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised central nervous system function.

Because of the possibility of severe or even fatal adverse reactions, patients should be informed not only of the benefits of therapy but also of the risks involved.

Roferon-A should be administered with caution to patients with cardiac disease or with any history of cardiac illness. No direct cardiotoxic effect has been demonstrated, but it is likely that acute, self-limited toxicities (*i.e.*, fever, chills) frequently associated with Roferon-A administration may exacerbate preexisting cardiac conditions. Rarely, myocardial infarction has occurred in patients receiving Roferon-A.

Caution should be exercised when administering Roferon-A to patients with myelosuppression.

Central nervous system adverse reactions have been reported in a number of patients. These reactions included decreased mental status, exaggerated central nervous system function, and dizziness. More severe obtundation and coma have been rarely observed. Most of these abnormalities were mild and reversible within a few days to three weeks upon dose reduction or discontinuation of Roferon-A therapy. Careful periodic neuropsychiatric monitoring of all patients is recommended.

Leukopenia and elevation of hepatic enzymes occurred frequently but were rarely dose-limiting. Thrombocytopenia occurred less frequently. Proteinuria and increased cells in urinary sediment were also seen infrequently. Rarely, significant hepatic, renal and myelosuppressive toxicities were noted.

PRECAUTIONS: *General:* In all instances where the use of Roferon-A is considered for chemotherapy, the physician must evaluate the need and usefulness of the drug against the risk of adverse reactions. Most adverse reactions are reversible if detected early. If severe reactions occur, the drug should be reduced in dosage or discontinued and appropriate corrective measures should be taken according to the clinical judgment of the physician. Reinstitution of Roferon-A therapy should be carried out with caution and with adequate consideration of the further need for the drug and alertness as to possible recurrence of toxicity.

The minimum effective dose of Roferon-A for treatment of hairy cell leukemia has not been established.

Information for Patient: Patients should be cautioned not to change brands of Interferon without medical consultation, as a change in dosage may result. Patients should be informed regarding the potential benefits and risks attendant to the use of Roferon-A. If home use is determined to be desirable by the physician, instructions on appropriate use should be given, including review of the contents of the enclosed Patient Information Sheet. Patients should be well hydrated, especially during the initial stages of treatment.

Laboratory Tests: Periodic complete blood counts and liver function tests should be performed during the course of Roferon-A treatment. They should be performed prior to therapy and at appropriate periods during therapy. Since responses of hairy cell leukemia are not generally observed for one to three months after initiation of treatment, very careful monitoring for severe depression of blood cell counts is warranted during the initial phase of treatment.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis and Impairment of Fertility:

Carcinogenesis: Roferon-A has not been tested for its carcinogenic potential.

Mutagenesis: A. Internal studies—Ames tests using six different tester strains, with and without metabolic activation, were performed with Roferon-A up to a concentration of 1920 µg/plate. There was no evidence of mutagenicity.

Human lymphocyte cultures were treated *in vitro* with Roferon-A at noncytotoxic concentrations. No increase in the incidence of chromosomal damage was noted.

B. Published studies—There are no published studies on the mutagenic potential of Roferon-A. However, a number of studies on the genotoxicity of human leukocyte interferon have been reported.

A chromosomal defect following the addition of human leukocyte interferon to lymphocyte cultures from a patient suffering from a lymphoproliferative disorder has been reported.

In contrast, other studies have failed to detect chromosomal abnormalities following treatment of lymphocyte cultures from healthy volunteers with human leukocyte interferon.

It has also been shown that human leukocyte interferon protects primary chick embryo fibroblasts from chromosomal aberrations produced by gamma rays.

Impairment of Fertility: Roferon-A has been studied for its effect on fertility in Macaca mulatta (rhesus monkeys). Nonpregnant rhesus females treated with Roferon-A at doses of 5 and 25 million IU/kg/day have shown menstrual cycle irregularities, including prolonged or shortened menstrual periods and erratic bleeding; these cycles were considered to be anovulatory. These monkeys returned to a normal menstrual rhythm following discontinuation of treatment.

Drug Interactions: Interactions between Roferon-A and other drugs have not been fully evaluated.

PREGNANCY: *Teratogenic Effects:* Pregnancy Category C. Roferon-A has been shown to demonstrate a statistically significant increase in abortifacient activity in rhesus monkeys when given at approximately 20 to 500 times the human dose. A study in pregnant rhesus monkeys treated with 1, 5 or 25 million IU/kg/day of Roferon-A in their early to midfetal period (days 22 to 70 of gestation) has failed to demonstrate teratogenic activity for Roferon-A. There are no adequate and well-controlled studies in pregnant women.
Nonteratogenic Effects: Dose-related abortifacient activity was observed in pregnant rhesus monkeys treated with 1, 5 or 25 million IU/kg/day of Roferon-A in their early to midfetal period (days 22 to 70 of gestation). A late-fetal period study (days 79 to 100 of gestation) is in progress and as yet there have been no reports of any increased rate of abortion.
Usage in Pregnancy: Safe use in human pregnancy has not been established. Therefore, Roferon-A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. Information from primate studies showed dose-related menstrual irregularities and an increased incidence of spontaneous abortions. Therefore, fertile women should not receive Roferon-A unless they are using effective contraception during the therapy period.

Male fertility and teratologic evaluations have yielded no significant adverse effects to date.
Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from Roferon-A, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.
Pediatric Use: Safety and effectiveness in children under 18 years of age have not been established.
ADVERSE REACTIONS: The following data on adverse reactions are based on the subcutaneous or intramuscular administration of Roferon-A as a single agent for the treatment of 63 patients with hairy cell leukemia during investigational trials in the United States.
Flu-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), myalgias (73%) and headache (71%) occurred in the majority of patients and tended to diminish with continuing therapy. Other side effects such as anorexia (46%), nausea (32%), emesis (10%), diarrhea (29%), dizziness (21%), rash (18%), change in taste (13%), dryness or inflammation of the oropharynx (16%), dry skin or pruritus (13%) and weight loss (14%) were observed with moderate frequency. Less commonly, diaphoresis (8%), paresthesias (6%), numbness (6%), partial alopecia (8%), reactivation of herpes labialis (8%), transient impotence (6%) and arthralgias (5%) were also observed. Rarely (<3%), central nervous system effects including decreased mental status, depression, visual disturbances, sleep disturbances and nervousness, as well as cardiac adverse events, including hypertension, chest pain, arrhythmias and palpitations, were reported. Adverse experiences which occurred rarely and may have been related to underlying disease, included epistaxis, bleeding gums, ecchymosis and petechiae. Miscellaneous adverse events, such as night sweats, urticaria, conjunctivitis and inflammation at the site of injection, were also rarely observed.
Roferon-A has also been evaluated for the treatment of many other types of cancer under investigational trials in the United States. These studies generally utilized higher doses (12 to 50 million IU/m^2). All of the previously described adverse reactions which occurred in patients with hairy cell leukemia were also observed in patients receiving higher doses. The incidence of most adverse reactions was similar between the two groups, but tended to be more severe in patients who received higher doses of Roferon-A. Some additional adverse effects which occurred in these patients included confusion (10%), hypotension (6%), lethargy (3%) and edema (3%). Adverse experiences occurring in less than 1% of these patients and observed only in patients with malignancies other than hairy cell leukemia are as follows: *Gastrointestinal*—abdominal fullness, hypermotility and hepatitis; *Central Nervous System*—gait disturbance, poor coordination, hallucinations, syncope, seizures, encephalopathy, psychomotor retardation, coma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, dysphasia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional lability, irritability, hyperactivity, involuntary movements, claustrophobia and loss of libido; *Peripheral Nervous System*—muscle contractions; *Cardiovascular*—congestive heart failure, pulmonary edema, myocardial infarction, Raynaud's phenomenon and hot flashes; *Pulmonary*—bronchospasm and tachypnea; *Miscellaneous*—excessive salivation and flushing of skin.
Abnormal Laboratory Test Values: The percentage of patients with hairy cell leukemia or with other types of malignancies who experienced a significant abnormal laboratory test value at least once during their treatment is shown in the following table:

ABNORMAL LABORATORY TEST VALUES

	Hairy Cell Leukemia (n = 63)	Overall Safety Summary[*] (n = 1019)
Hematologic		
Leukopenia	59%	69%
Neutropenia	39%	58%
Thrombocytopenia	42%	42%
Decreased Hemoglobin	36%	6.3%
Decreased Hematocrit	43%	12.5%
Mean Time to Nadir		
WBC	38 days	22 days
Platelets	19 days	17 days
Hepatic		
SGOT	47%	78%
Alkaline Phosphatase	18%	48%
LDH	12%	47%
Bilirubin	1.6%	31%
Renal/Urinary		
BUN	2%	10%
Serum Creatinine	3%	10%
Uric Acid[†]	6%	15%
Proteinuria[†]	10%	25%
Other Chemistry Tests		
Hypocalcemia	10%	51%
Elevated FBS[‡]	33%	39%
Elevated Serum Phosphorus	2%	17%

[*] Patients with hairy cell leukemia are included in this overall summary.
[†] The majority of abnormal uric acid values and proteinuria were of minimal severity. Since these abnormalities are commonly observed in patients with advanced malignancy, it is difficult to ascertain their relationship to Roferon-A treatment.
[‡] Random glucose determination.

Neutralizing antibodies to Roferon-A were detected in approximately 27% of all patients (3.4% for patients with hairy cell leukemia). No clinical sequelae of their presence have been documented thus far. Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.[4]
DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The induction dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as a subcutaneous or intramuscular injection. Subcutaneous administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count <50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, three times per week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended.
Patients should be treated for approximately six months before the physician determines whether to continue therapy in patients who respond or discontinue therapy in patients who did not respond. Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.
If severe reactions occur, dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve. The minimum effective dose of Roferon-A has not been established.
DIRECTIONS FOR USE: The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are thrombocytopenic (platelet count <50,000) or who are at risk for bleeding.
Injectable Solution:
3 million IU Roferon-A per vial—Each 1 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative.
18 million IU Roferon-A per vial (for multiple-dose use)—Each 1 mL contains 6 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative. Each 0.5 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche.
The injectable solution should be stored in the refrigerator at 36° to 46°F (2° to 8°C). Do *not* freeze. Do not shake.
Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.
HOW SUPPLIED: Sterile Roferon-A is supplied as single and multiple-dose vials:
Roferon-A Injectable Solution: Vials containing 3 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/mL). Boxes of 10 (NDC 0004-1987-01).
Roferon-A Injectable Solution: Multiple-dose vials containing 18 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/0.5 mL). Boxes of 1 (NDC 0004-1988-09).

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2. Foon KA *et al*: *Blood 64*(Suppl. 1):164a, 1984.
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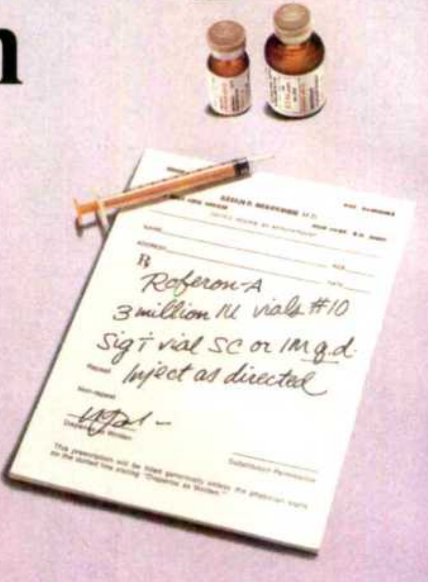
4. Jones GJ, Itri LM: *Cancer 57*(Suppl.):1709-1715, 1986.

THIS PRODUCT INFORMATION ISSUED JUNE 1986

ROCHE
Roche Laboratories
Division of Hoffmann-La Roche Inc.
Nutley, New Jersey 07110

Dosing simple enough for self-injection



℞
Roferon-A
3 million IU vials #10
Sig ī vial SC or IM q.d.
Inject as directed

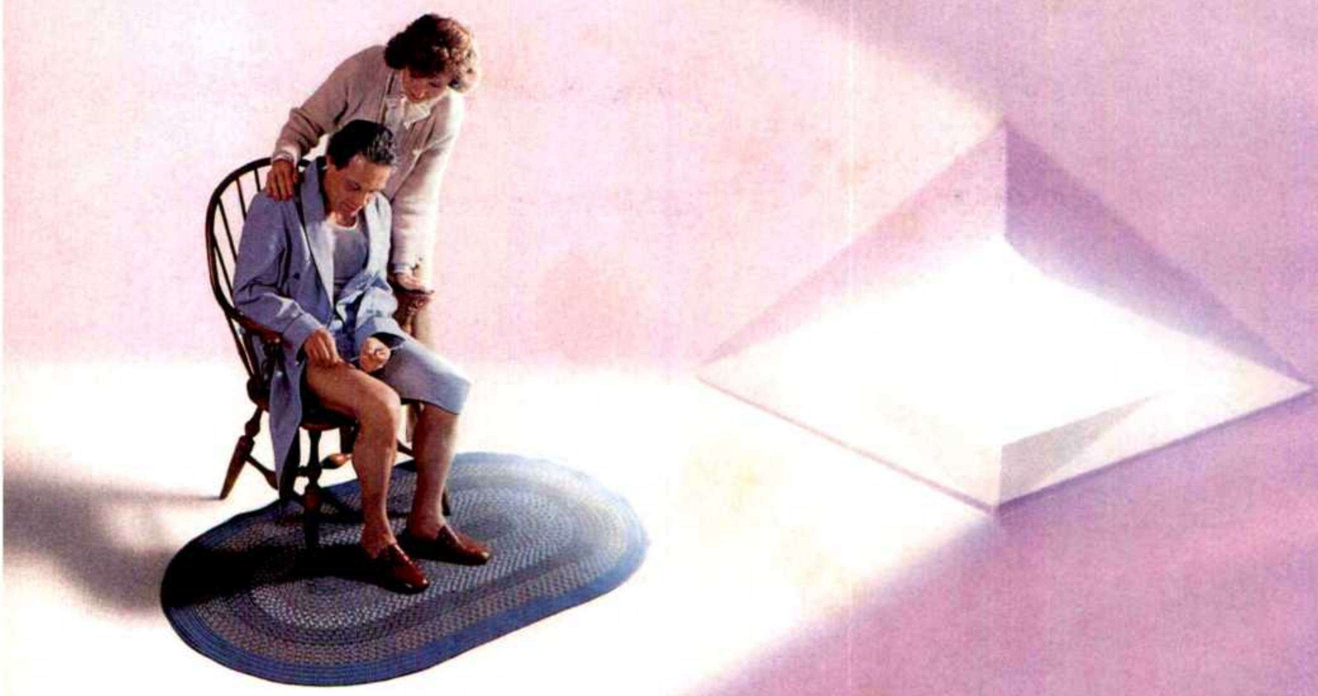
Available as ready-to-use injectable solution; no mixing or reconstitution necessary

Fixed dose means no weight or body surface area calculation necessary; saves time; reduces potential for error and waste

SC or IM injection offers convenient administration in home, office, clinic or hospital

Allows administration by self or family member

Caution patients not to change brands of Interferon without medical consultation, as a change in dosage may result.



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ROFERON®-A

Interferon BRAND OF alfa-2a, recombinant/Roche

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New! The latest edition of an authoritative reference book . . .

RED CELL METABOLISM

A MANUAL OF BIOCHEMICAL METHODS

Third Edition

ERNEST BEUTLER, M.D.

This third edition of **Red Cell Metabolism: A Manual of Biochemical Methods** presents a detailed discussion of the methods required for the measurement of red cell enzymes and metabolic intermediates. The techniques described—which have evolved after years of performing these assays in a laboratory environment—are essential both to the clinical diagnosis of certain types of anemia and to basic research on red blood cells. Topics covered include the fundamentals of handling blood samples, methods of reagent preparation, instrumentation, and calculation of results. New methods, improved procedures, and the increasing importance of certain red cell constituents such as GSH-S-transferase, superoxide dismutase, Na^+-K^+-ATPase, phosphoglycolate phosphatase, and creatine are among the factors that have brought about the need for this updated edition of the text.

Written for hematologists, clinical pathologists, biochemists, and cellular biologists, the third edition of **Red Cell Metabolism** represents the most authoritative and current information in the field of red cell enzymology, and is internationally recognized as the classic text on the subject.

CONTENTS:

Introduction: Purpose and Organization of the Manual. Red Cell Metabolism. **Basic Techniques and Equipment:** The Preparation of Red Cells for Assay. Reagents. Instrumentation. Calculation of Results. **Red Cell Glycolytic Enzymes:** Hexokinase (Hx). Glucose Phosphate Isomerase (GPI). Phosphofructokinase (PFK). Aldolase. Triose Phosphate Isomerase (TPI). Glyceraldehyde Phosphate Dehydrogenase (GAPD). Phosphoglycerate Kinase (PGK). Diphosphoglycerate Mutase (DPGM). Monophosphoglyceromutase (MPGM). Enolase. Pyruvate Kinase (PK). Lactate Dehydrogenase (LDH). **Red Cell Hexose Monophosphate Shunt and Other Enzymes:** Glucose-6-Phosphate Dehydrogenase (G-6-PD) and 6-Phosphogluconate Dehydrogenase (6-PGD). Glutathione Reductase (GR). Glutathione Peroxidase (GSH-Px). Glutathione S-Transferase. NADPH Diaphorase. NADH Methemoglobin Reductase (NADH-Ferricyanide Reductase). Superoxide Dismutase. Phosphoglucomutase (PGM). Glutamate-Oxaloacetate Transaminase (GOT). Phosphoglycolate Phosphatase. Adenylate Kinase (AK). Na^+-K^+ATPase and Mg^{++}ATPase. Adenosine Demmainase (AD). Pyrimidine 5′ Nucleotidase. Acetylcholinesterase. Catalase. Galactose-1-Phosphate Uridyl Transferase. Galactokinase (GK). UDP Glucose-4-Epimerase (Epimerase). **Intermediate Compounds:** ATP. AMP and ADP. 2,3-Diphosphoglycerate (2,3-DPG). Reduced Glutathione (GSH). Oxidized Glutathione (GSSG). Creatine. Glucose. Pyruvate. Lactate. Glucose-6-P and Fructose-6-P. Glyceraldehyde-3-P (GAP), Dihydroxyacetone-P (DHAP), and Fructose-DiP (FDP). 3-Phosphoglyceric Acid (3-GPA). 2-Phosphoglyceric Acid (2-PGA) and Phosphoenolpyruvate. **Screening Techniques:** General Principles. Glucose Phosphate Isomerase Screening Test. Triose Phosphate Isomerase Screening Test. Pyruvate Kinase Screening Test. Glucose-6-Phosphate Dehydrogenase Screening Test. Glutathione Reductase Screening Test. NADH Methemoglobin Reductase (NADH Diaphorase). Pyrimidine 5′ Nucleotidase Screening Test. **Appendix 1:** Activities of Red Cell Enzymes in Normal Adults. **Appendix 2:** Effect of Temperature on Red Cell Enzyme Assays. **Appendix 3:** Levels of Intermediates in Red Cells of Normal Adults. **Appendix 4:** Frequently Used Molecular Weights.

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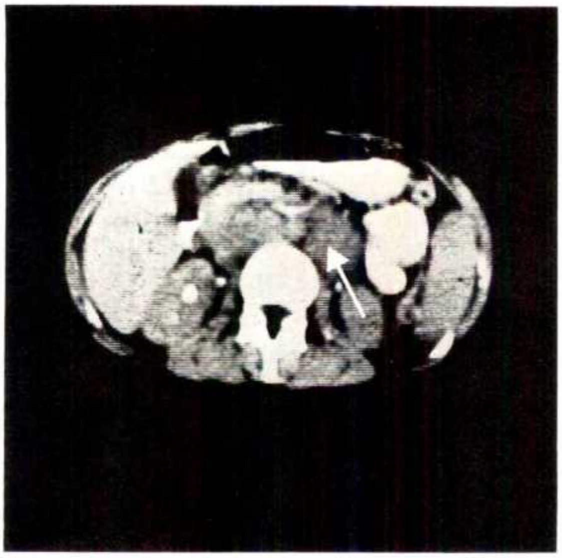
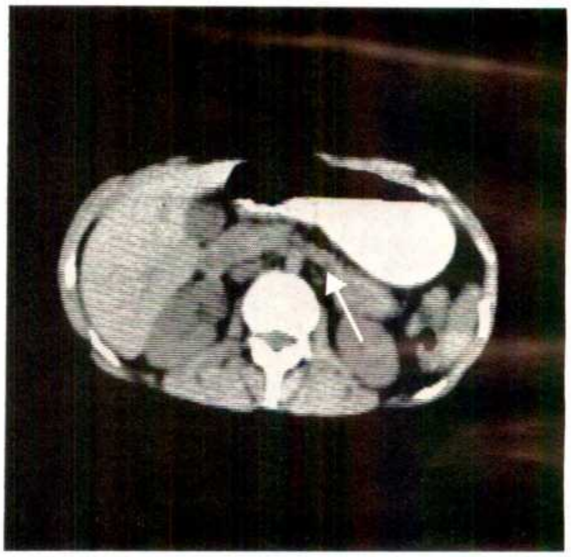


Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al:* Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al:* Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

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*Case information on file at Bristol-Myers Oncology Division

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M-BACOD

4. Skarin AT, Canellos GP, Rosenthal DS, *et al:* Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
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m-BACOD

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MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

Blenoxane® *(sterile bleomycin sulfate)*

Brief Summary of Prescribing Information (1) 10 84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents.

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: **Pulmonary** – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain $FI\ O_2$ at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

SUPPLY: Each vial contains 15 units of Blenoxane as sterile bleomycin sulfate. NDC 0015-3010-20

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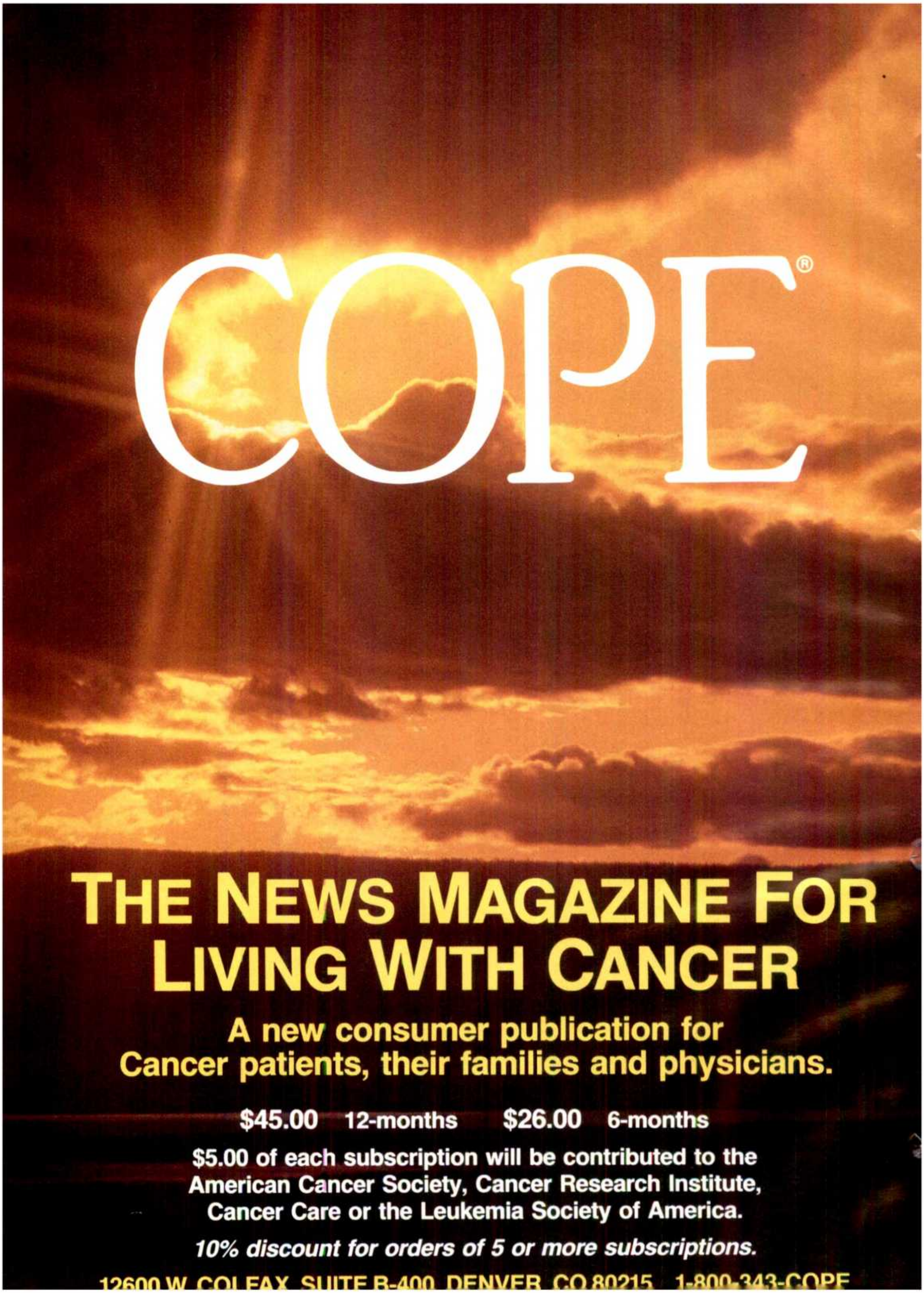

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Some patients who need intravenous gamma globulin therapy cannot tolerate IgA. Life-threatening anaphylactic reactions have resulted from infusion of immune globulin containing only *15 micrograms of IgA per milliliter.** Our response: A DEAE-Sephadex adsorption step is included in the GAMMAGARD® IGIV manufacturing process; this removes most non-IgG proteins and IgA.

Result: IgA levels in GAMMAGARD® IGIV are very low; radioimmunoassay test results for six randomly selected lots range between *0.27 and 1.55 micrograms/ml.* This degree of purity greatly reduces the risk of side reactions, particularly for highly sensitive patients. On-going studies (as reported in *The New England Journal of Medicine**), indicate that patients with long histories of IgA-related anaphylaxis tolerate GAMMAGARD® IGIV well. IGIV should still be given with caution in these patients.

The "second-generation" process used to produce GAMMAGARD® IGIV is responsible for its excellent therapeutic characteristics. For example, GAMMAGARD® IGIV retains full native antibody activities, as well as the normal distribution of IgG subclasses.

When your immunocompromised patients require protection, think of GAMMAGARD® IGIV–the IGIV with very low levels of IgA, high levels of purified IgG monomer, and remarkably high titers for an impressive variety of pathogens–in short the IGIV with the purity, safety and efficacy your patients must have.

***Burks, A. W., Sampson, H. A., and Buckley, R. H. (1986):** *Anaphylactic Reactions after Gamma Globulin Administration in Patients with Hypogammaglobulinemia.* **New England Journal of Medicine** 314 (9): 560-564

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Artist's conception of DEAE-Sephadex adsorption

DIRECTION INSERT

Immune Globulin Intravenous (Human)
GAMMAGARD®

DESCRIPTION

Immune Globulin Intravenous (Human), GAMMAGARD® * is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8±0.4.

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average, the distribution of IgG subclasses present in this product is the same as is present in normal plasma.[1] Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from plasma units found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum levels is, therefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum proteins.[2,3] Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.[2,3,4,5]

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5]

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

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2. Waldmann TA, Storber W: Metabolism of immunoglobulins. *Prog Allergy 13*: 1-110, 1969

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CLINICAL PHARMACOLOGY

Gamimune N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 40% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune N equals or exceeds the three week half life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune N. It is not known whether Gamimune N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune N. Symptoms reported with Gamimune N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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Personal communications and references to publications in press by authors other than those submitting the paper must be substantiated by a letter from the investigator(s) concerned confirming the data or observations and granting the authors permission to cite the material.

EXAMPLES OF REFERENCES

Journal article, one or more authors:

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8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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HERPES SIMPLEX INFECTION IN THE COMPROMISED HOST:

OFTEN SERIOUS, TOO OFTEN UNSUSPECTED

Number one in a series of typical case presentations.

Esophagitis in a leukemia patient

In patients with hematologic malignancies, the visceral organ most frequently involved by herpes simplex virus (HSV) infection is the esophagus.[1] Extensive ulcerative lesions may develop on the upper two thirds of the esophagus with confluent lesions of the distal third.[2] In some cases, however, the mucosa may be grossly unremarkable even when microscopic examination shows evidence of herpetic ulcerative esophagitis.[1] Similarly, some patients may display symptoms of dysphagia and burning retrosternal pain,[3] whereas others may appear asymptomatic.[1] The diagnostic method of choice for patients with suspected herpetic esophagitis is esophagoscopy with visualization of the mucosa and procurement of cytologic and biopsy specimens from ulcerated areas.[1]

References: 1. Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **2.** Wong KK, Hirsch MS: Herpes virus infections in patients with neoplastic disease. Diagnosis and therapy. *Am J Med* 1984; 76:464-478. **3.** Lightdale CJ, Wolf DJ, Marcucci RA, et al: Herpetic esophagitis in patients with cancer: Ante mortem diagnosis by brush cytology. *Cancer* 1977;39:223-226.

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Herpetic infections may result in extensive disease and death

Immunocompromised patients are highly vulnerable to HSV infection. Such infections may interrupt therapy and even lead to death if not detected early and managed aggressively.[1] Although the initial appearance may be mild, HSV infection may progress to severe localized mucocutaneous ulcerations. The greatest threat that herpes simplex poses is an infection that disseminates from a mucocutaneous or unrecognized primary lesion and involves the lungs, liver, esophagus, or central nervous system.[1] Furthermore, herpetic ulcers frequently provide an entry for secondary bacterial and fungal pathogens.[2]

Infections may not be diagnosed correctly without virologic study

Some investigators claim that the actual incidence of serious HSV infection is probably higher than reported because "the possibility of herpetic disease is insufficiently considered and appropriate diagnostic viral studies are often not carried out."[3] One study, for example, found histocytologic evidence of visceral herpes infection in 56 patients even though herpetic involvement was not suspected in any of the patients prior to autopsy.[2]

ZOVIRAX I.V. halts viral replication and speeds healing even in debilitated patients

ZOVIRAX I.V. has been shown to be a highly effective treatment for severe mucocutaneous HSV infection in immunocompromised patients. In controlled trials, ZOVIRAX I.V. rapidly halted viral shedding — within 3 days in most patients — and significantly shortened healing time as well as duration of pain.[4,5] Furthermore, ZOVIRAX I.V. may allow patients to return home sooner and enable patients with intraoral lesions to resume normal eating habits.[6]

ZOVIRAX I.V. is well tolerated by immunocompromised patients

A multicenter collaborative trial of ZOVIRAX I.V. in immunocompromised patients reported that: "Clinical benefits were obtained without appreciable toxicity. The most common reactions were irritation of peripheral veins used for drug infusion and a low incidence of rash.... Significant renal or hematologic toxicity did not occur, even in these seriously ill patients who were commonly receiving other drugs concomitantly."[5]

Note: Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes. Please see brief summary for more information.

References: **1.** Feldman S, Cox F: Viral infections and hematological malignancies. *Clin Haematol* 1976;5:311-328. **2.** Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **3.** Muller SA, Herrmann EC, Winkelmann RK: Herpes simplex infections in hematologic malignancies. *Am J Med* 1972;52:102-114. **4.** Wade JC, Newton B, McLaren C, et al: Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation. *Ann Intern Med* 1982;96:265-269. **5.** Meyers JD, Wade JC, Mitchell CD, et al: Multicenter collaborative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. *Am J Med* 1982;73(1A):229-235. **6.** Data on file, Burroughs Wellcome Co.

An effective response to a life-threatening infection
ZOVIRAX® I.V. INFUSION
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An effective response to a life-threatening infection

ZOVIRAX® I.V. INFUSION (acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M² every 8 hours (750 mg/M²/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously, or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M²) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M² at a constant rate over 1 hour, every 8 hours (750 mg/M²/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M²)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

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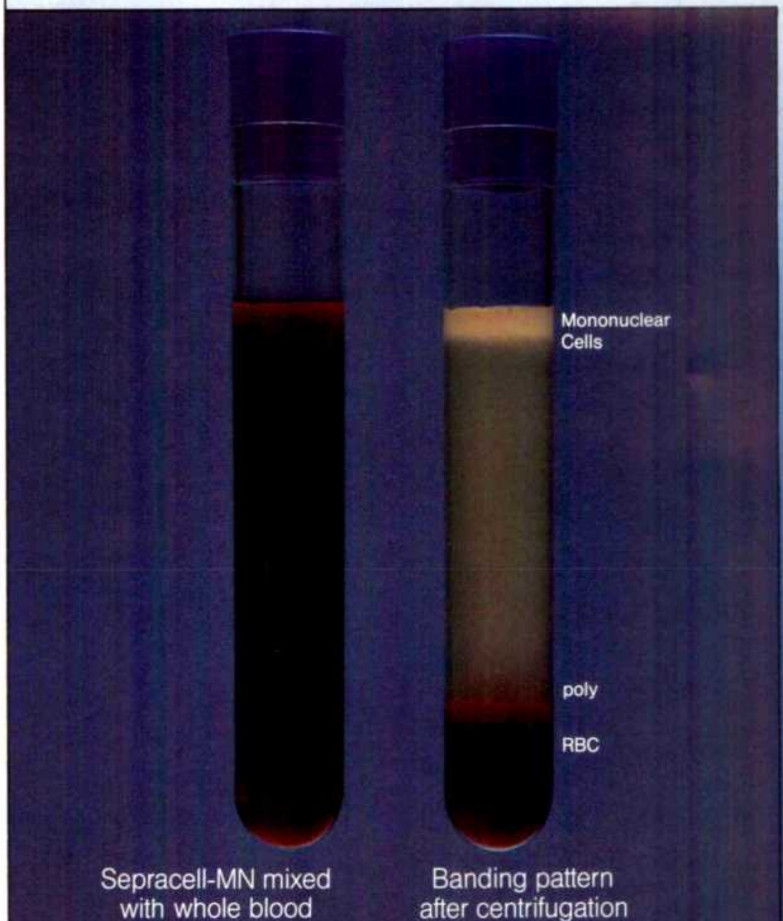

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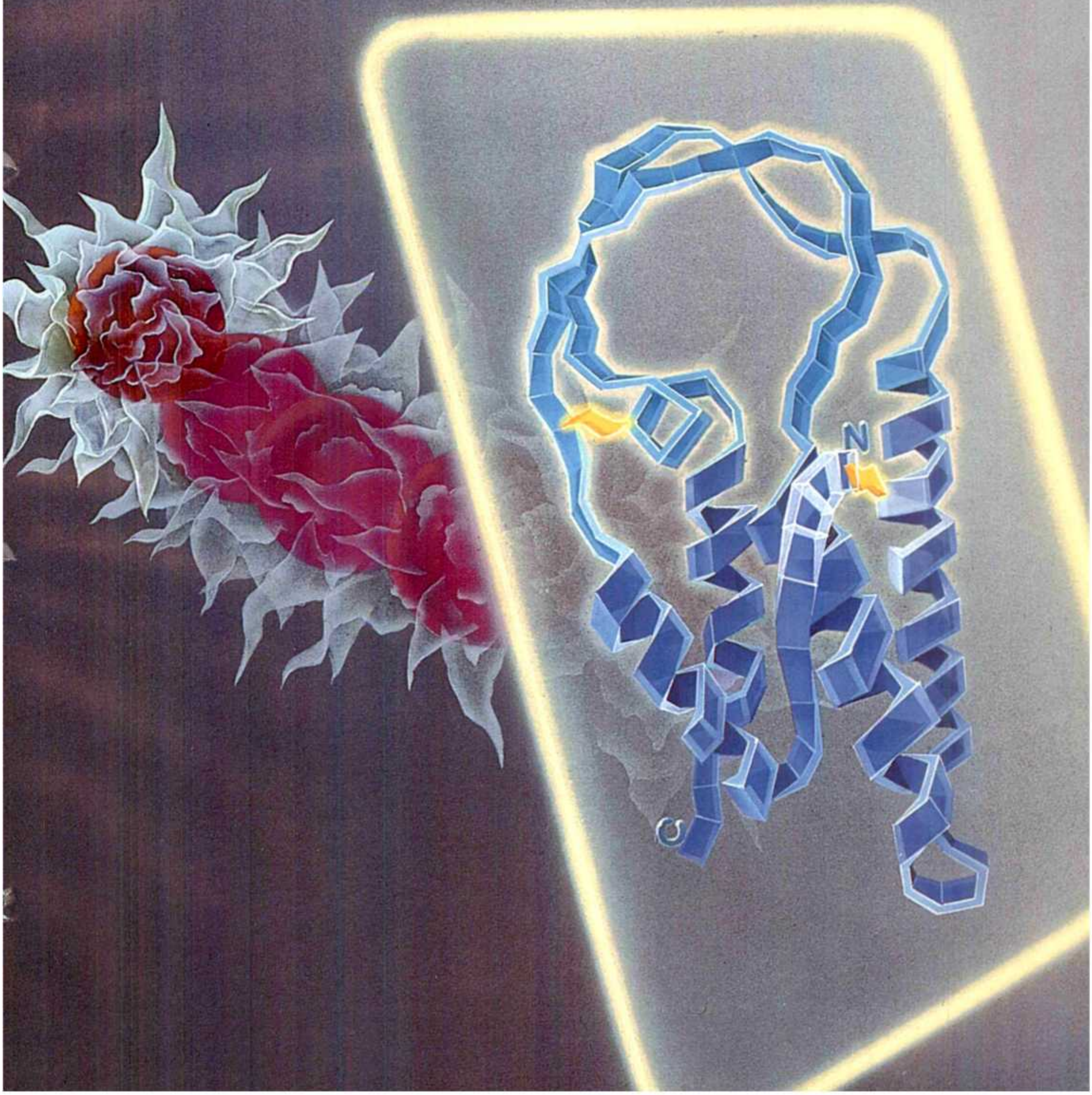

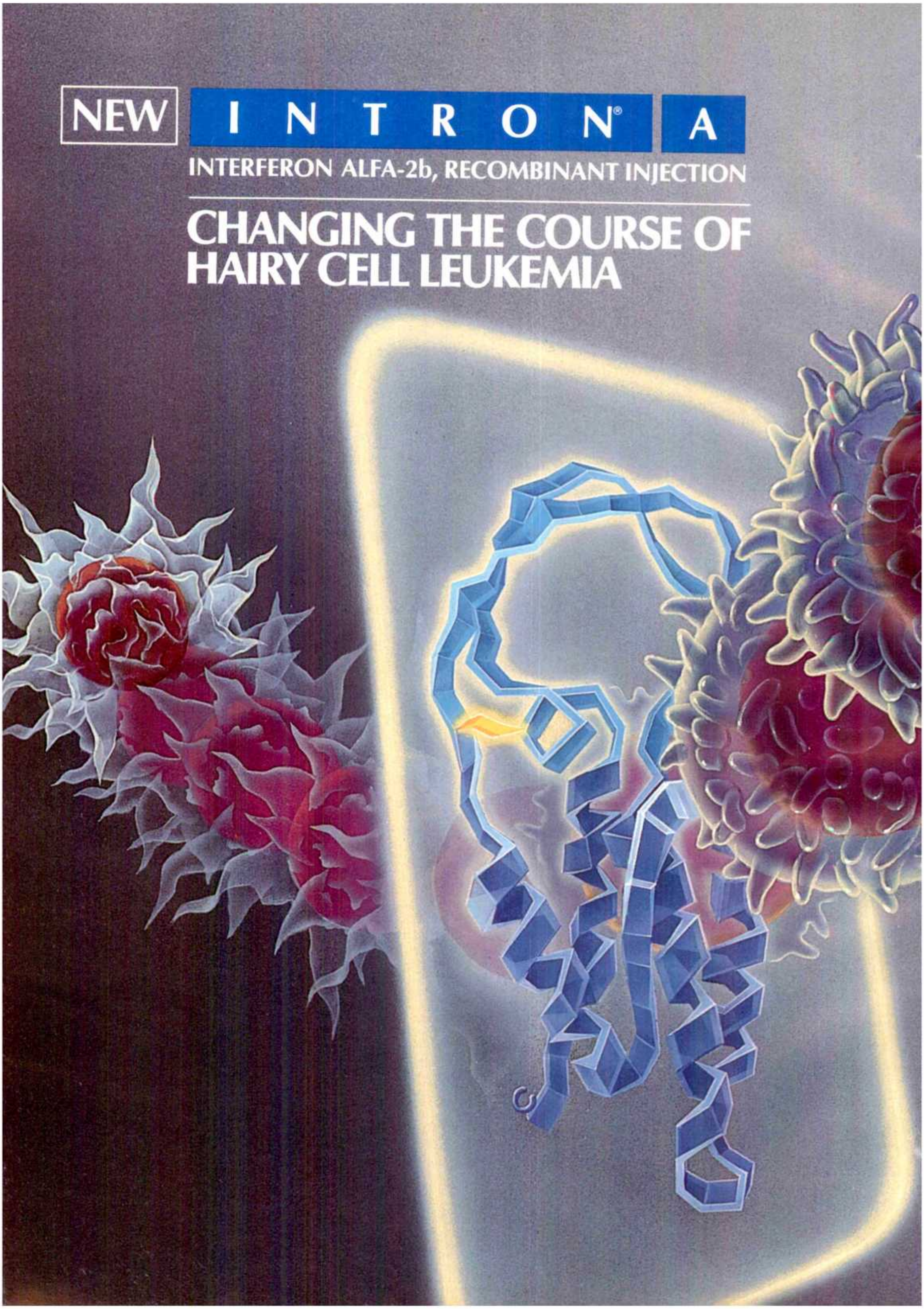

NEW INTRON® A

INTERFERON ALFA-2b, RECOMBINANT INJECTION

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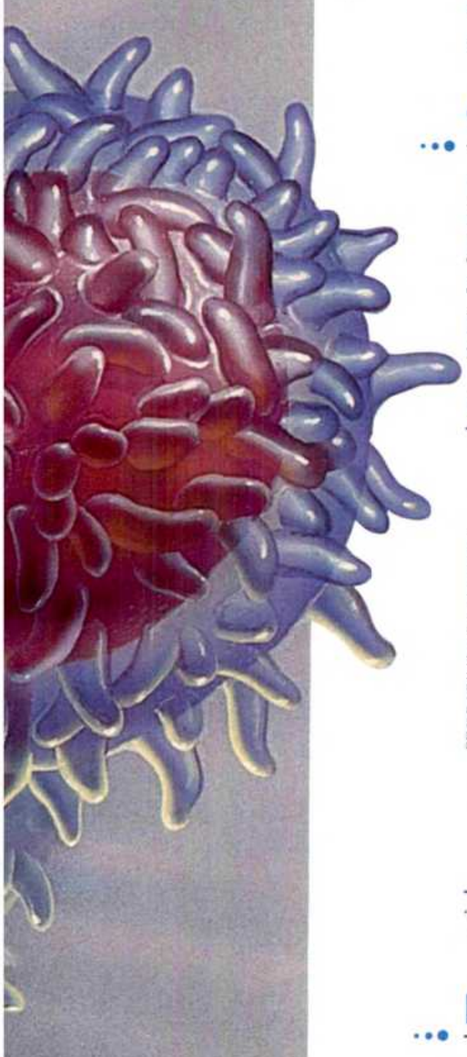

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The efficacy of INTRON A was established in a 26-center clinical trial of 145 patients with progressive hairy cell leukemia, a B-cell malignancy.[1,2,3] INTRON A achieved a 74% objective response, measured as normalization of granulocyte, hemoglobin and platelet values and elimination of transfusion dependency.[1]

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In a separate analysis, a 24-month comparison was conducted between the INTRON A study population and historical controls.* After three months of therapy, patients receiving INTRON A reached and maintained a plateau of 92% probability of survival. The probability of survival among historical controls, however, declined steadily to 40% at 24 months.[1]

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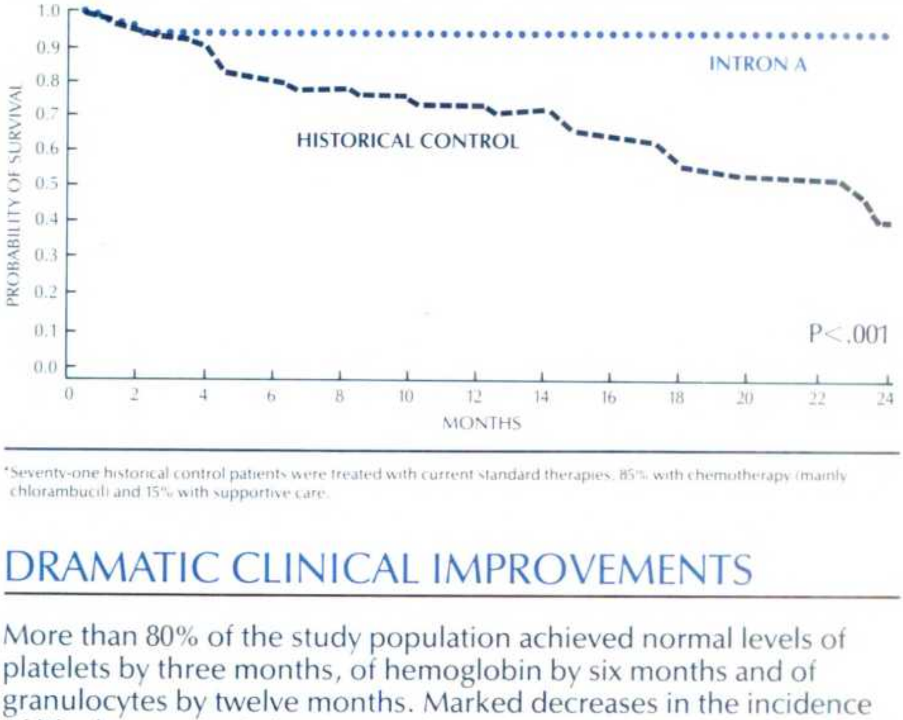


*Seventy-one historical control patients were treated with current standard therapies: 85% with chemotherapy (mainly chlorambucil) and 15% with supportive care.

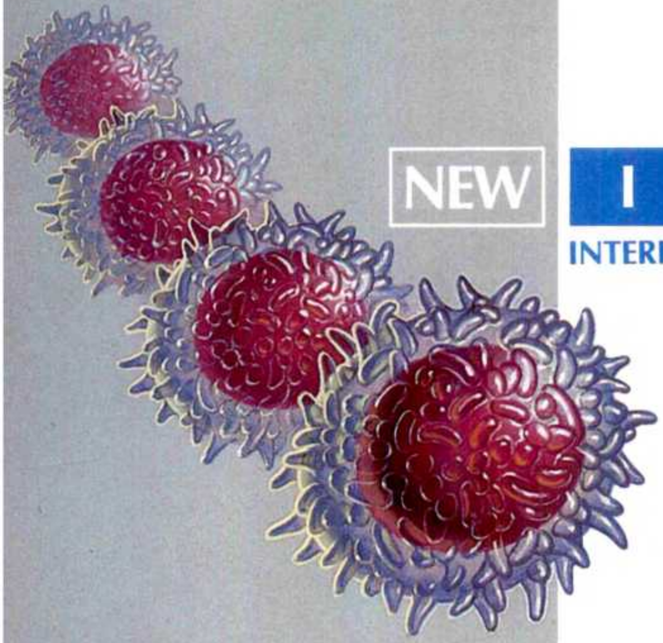
DRAMATIC CLINICAL IMPROVEMENTS

More than 80% of the study population achieved normal levels of platelets by three months, of hemoglobin by six months and of granulocytes by twelve months. Marked decreases in the incidence of life-threatening infections and transfusion dependency preceded normalization of hematologic values.[1]

THERAPY WELL TOLERATED

Treatment-related side effects were generally mild to moderate and rapidly reversible upon discontinuation of therapy. The predominant complaint was a flu-like syndrome, which usually diminished with continued therapy. Mild-to-moderate skin rashes occurred intermittently in 40% of the study population. A low incidence of mild-to-moderate GI disorders was also reported.[1]

For a brief summary of prescribing information, please see the following page.



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PRODUCT INFORMATION

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INTRON® A Interferon alfa-2b recombinant for Injection

BRIEF SUMMARY

INDICATIONS AND USAGE INTRON A for Injection is indicated for the treatment of patients 18 years of age or older with hairy cell leukemia. Studies have shown that INTRON A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and non-splenectomized patients.

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically during treatment to determine whether response to treatment has occurred. If a patient does not respond within 6 months, treatment should be discontinued. If a response to treatment does occur, treatment usually should be continued until no further improvement is observed and these laboratory parameters have been stable for about 3 months (see **DOSAGE AND ADMINISTRATION**). It is not known whether continued treatment after that time point is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS INTRON A is contraindicated in patients with a history of hypersensitivity to interferon alfa or any component of the injection.

WARNINGS Moderate to severe adverse experiences may require modification of the patient's dosage regimen, or in some cases, termination of therapy with INTRON A.

Because of the fever and other "flu-like" symptoms associated with INTRON A administration, it should be used cautiously in patients with debilitating medical conditions, such as those with a history of cardiovascular disease (e.g., unstable angina, uncontrolled congestive heart failure), pulmonary disease (e.g., chronic obstructive pulmonary disease), or diabetes mellitus prone to ketoacidosis. Caution should also be observed in patients with coagulation disorders (e.g., thrombophlebitis, pulmonary embolism) or severe myelosuppression.

Patients with platelet counts of less than $50{,}000/mm^3$ should not be administered INTRON A intramuscularly, but instead by subcutaneous administration.

Cardiovascular adverse experiences which include significant hypotension, arrhythmia, or tachycardia of 150 beats per minute or greater, were observed in approximately 3% of the patients studied who had various malignancies and were treated at doses higher than those for hairy cell leukemia. The incidence of these complications in patients with preexisting heart disease is unknown. Hypotension may occur during administration, or up to two days post therapy, and may require supportive therapy including fluid replacement to maintain intravascular volume. Supraventricular arrhythmias occurred rarely and appeared to be correlated with preexisting conditions, and prior therapy with cardiotoxic agents. These adverse experiences were controlled by modifying the dose or discontinuing treatment, but may require specific additional therapy.

Those patients with a recent history of myocardial infarction and/or previous or current arrhythmic disorder, who require INTRON A therapy, should be closely monitored (see **Laboratory Tests**).

Central nervous system effects manifested by depression, confusion and other alterations of mental status were observed in about 2% of hairy cell leukemia patients treated with INTRON A. The overall incidence in a larger patient population with other malignancies treated with higher doses of INTRON A was 10%. More significant obtundation and coma have been observed in some patients, usually elderly, treated at higher doses for other malignant diseases. These effects are usually rapidly reversible. In a few severe episodes, full resolution of symptoms has taken up to three weeks. Patients should be closely monitored until resolution of these effects. Discontinuation of INTRON A Interferon alfa-2b, recombinant for Injection therapy may be required. Narcotics, hypnotics, or sedatives may be used concurrently with caution.

Acute serious hypersensitivity reactions (e.g., urticaria, angioedema, bronchoconstriction, anaphylaxis, etc.) have not been observed in patients receiving INTRON A; however, if such an acute reaction develops, the drug should be discontinued immediately and appropriate medical therapy instituted. Transient cutaneous rashes have occurred in some patients following injection, but have not necessitated treatment interruption.

Laboratory abnormalities which occurred in hairy cell leukemia patients included elevated SGOT and SGPT, which occurred in 4% and 13% of patients, respectively. The overall incidences of laboratory abnormalities in a larger patient population with other malignancies, and treated at higher doses were somewhat higher. These included elevated liver function tests (SGOT, SGPT in 10% of patients) and reductions in granulocyte (20% of patients) and platelet counts (18% of patients) (see **Laboratory Tests**). These abnormalities are usually mild to moderate and transient. Severe abnormalities of these laboratory parameters are usually rapidly reversible upon cessation or reduction of INTRON A therapy.

PRECAUTIONS Information for Patients Patients being treated with INTRON A should be directed in its appropriate use, informed of benefits and risks associated with treatment and referred to the Patient Information Sheet.

Patients should be cautioned not to change brands of Interferon without medical consultation as a change in dosage may result.

It is advised that patients be well hydrated especially during the initial stages of treatment.

Laboratory Tests In addition to those tests normally required for monitoring patients with hairy cell leukemia, the following laboratory tests are recommended for all patients on INTRON A therapy, prior to beginning treatment and then periodically thereafter:

- Standard hematologic tests — including complete blood counts and differential as well as platelet counts.
- Blood chemistries — electrolytes and liver function tests.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer, should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis, Impairment of Fertility Studies with INTRON A have not been performed to determine carcinogenicity, or the effect on fertility. Interferon may impair fertility. Mutagenicity studies with INTRON A revealed no adverse effects.

Studies in mice, rats, and monkeys injected with INTRON A for up to one month have revealed no evidence of toxicity. However, due to the known species-specificity of interferon, the effects in animals are unlikely to be predictive of those in man.

Pregnancy Category C Animal reproduction studies have not been conducted with INTRON A. It is also not known whether INTRON A can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. INTRON A should be given to a pregnant woman only if clearly needed. Another interferon alfa preparation has been shown to have abortifacient effects in *Macaca mulatta* (rhesus monkeys) when given at 20 to 500 times the human dose. Therefore, INTRON A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. In studies of interferon use in non-human primates, abnormalities of the menstrual cycle have been observed.

INTRON A Interferon alfa-2b, recombinant for Injection should be used with caution in fertile men and women.

Nursing Mothers It is not known whether this drug is excreted in human milk. However, studies in mice have shown that mouse interferons are excreted into the milk. Because of the potential for serious adverse reactions from INTRON A in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use Safety and effectiveness have not been established in patients below the age of 18 years.

ADVERSE REACTIONS Adverse reactions to INTRON A are dose-related. In studies of patients with malignancies other than hairy cell leukemia, hematologic and hepatic toxicities were more common in patients receiving individual injections greater than 10 million IU, than in patients receiving less than this amount. Hematologic, hepatic, cardiovascular and neurologic toxicities were more common in patients receiving more than 30 million IU per injection than in patients receiving less than that amount.

The following adverse reactions were reported to be possibly or probably related to INTRON A therapy during clinical trials. The most frequently reported adverse reactions were flu-like symptoms, primarily fever, fatigue, and chills which occurred in almost all patients treated. Other organ systems in which reactions occurred in more than 5% of the patients are:

General — Taste alteration, anorexia and weight decrease occurred uncommonly.

Cardiovascular — Mild hypotension was frequently recorded although it was rarely symptomatic. Hypertension also occurred less commonly. Tachycardias occurred uncommonly usually in association with high fevers.

Gastrointestinal System — Nausea was common, although vomiting occurred rarely. Mild diarrhea was reported less commonly.

Hematologic — Mild thrombocytopenia and transient granulocytopenia occurred commonly.

Musculo-Skeletal System — Myalgia and arthralgia occurred commonly, usually in association with other flu-like symptoms.

Nervous System — Headache, somnolence, confusion, and dizziness occurred uncommonly. Ataxia and paresthesia were rare.

Psychiatric — Anxiety, depression and nervousness occurred uncommonly.

Skin and Appendages — Mild pruritus occurred uncommonly. Mild alopecia was uncommon. Various transient, mild skin rashes were seen frequently in patients with hairy cell leukemia.

Other Adverse Reactions — The following adverse reactions were reported with an incidence of 5% or less: leg cramps, constipation, insomnia, herpetic eruptions, non-herpetic cold sores, urticaria, hot flashes, supraventricular arrhythmias, epistaxis, stomatitis, paralytic ileus, dehydration, coagulation disorder (elevated PT and PTT), abnormal vision, tremor, emotional lability, chest pain, pharyngitis and syncope.

Adverse reactions reported rarely (less than 1%) were dyspepsia, purpura, dyspnea, sneezing, oculomotor paralysis, nasal congestion, flatulence, increased saliva, hyperglycemia, and ulcerative stomatitis.

Laboratory Value Changes — Those patient laboratory values which were normal to moderately abnormal (WHO grades 0–2) at baseline that worsened to either severe or life-threatening abnormalities (WHO grades 3 or 4) during some phase of treatment included: WBC, platelets, granulocytes, SGOT, creatinine, SGPT, LDH, and alkaline phosphatase.

DOSAGE AND ADMINISTRATION The recommended dosage of INTRON A for Injection for the treatment of hairy cell leukemia is 2 million IU/m^2 administered intramuscularly (see **WARNINGS**) or subcutaneously 3 times a week. Higher doses are not recommended. The normalization of one or more hematologic variables usually begins within 2 months of initiation of therapy. Improvement in all three hematologic variables may require 6 months or more of therapy.

This dosage regimen should be maintained unless the disease progresses rapidly, or severe intolerance is manifested. If severe adverse reactions develop, the dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. If persistent or recurrent intolerance develops following adequate dosage adjustment, or disease progresses, treatment with INTRON A Interferon alfa-2b, recombinant for Injection should be discontinued. The minimum effective dose of INTRON A has not been established.

At the discretion of the physician, the patient may self-administer the dose. The medication may be administered at bedtime.

HOW SUPPLIED INTRON A Interferon alfa-2b, recombinant for Injection is available in 3-, 5-, 10- and 25-million IU vials, each packaged with its own diluent.

Store INTRON A for Injection, both before and after reconstitution, between 2° and 8°C (36° and 46°F).

JBS-13762805

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50 mg/vial for I.M. use

DESCRIPTION: Each 1 ml ampul of Leucovorin Calcium *Lederle* contains Leucovorin 3 mg as the calcium salt which is the form preferred for intramuscular injection. Preservative: benzyl alcohol 0.9% w/v. The inactive ingredients are sodium chloride 0.56% w/v, Water for Injection qs 100% and sodium hydroxide or hydrochloric acid is used to adjust the pH to approximately 7.7.

Each 50 mg vial of Leucovorin Calcium cryodesiccated powder when reconstituted with 5 ml of sterile diluent contains Leucovorin 10 mg per ml as the calcium salt which is the form preferred for intramuscular injection. Contains no preservative. Dilute only with Bacteriostatic Water for Injection USP which contains benzyl alcohol. The inactive ingredients are sodium chloride 40 mg/vial, and sodium hydroxide or hydrochloric acid qs to pH approximately 8.1. When reconstituted as directed, the resulting solution must be used within seven days. If the product is reconstituted with Water for Injection USP, use immediately.

ACTIONS: Leucovorin (folinic acid) is the formyl derivative and active form of folic acid.

Leucovorin Calcium is useful clinically in circumventing the action of folate reductase. There is no evidence that intramuscular doses of greater than 1 mg have greater efficacy than those of 1 mg.

INDICATIONS: Indicated (a) to diminish the toxicity and counteract the effect of inadvertently administered overdosages of folic acid antagonists. (See Warnings) (b) In the treatment of the megaloblastic anemias due to sprue, nutritional deficiency, pregnancy, and infancy when oral therapy is not feasible.

CONTRAINDICATIONS: Not to be administered for the treatment of pernicious anemia or other megaloblastic anemias where Vitamin B_{12} is deficient.

WARNINGS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to lack of Vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

In the treatment of overdosage of folic acid antagonists, Leucovorin must be administered within one hour, if possible, and is usually ineffective if administered after a delay of four hours.

PRECAUTIONS: In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive.

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

DOSAGE: Megaloblastic anemia. No more than or up to 1 mg daily. There is no evidence that intramuscular doses greater than 1 mg daily have greater efficacy than those of 1 mg, additionally, loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg.

For the treatment of overdosage of folic acid antagonists. To be given in amounts equal to the weight of the antagonist given.

HOW SUPPLIED:
NDC 0205-4004-51—6—1 ml Ampuls
NDC 0205-5330-92—50 mg Vial of Cryodesiccated powder

LEDERLE PARENTERALS, INC
Carolina, Puerto Rico 00630
Rev 11/80

LEUCOVORIN CALCIUM TABLETS

BRIEF SUMMARY

Before prescribing Leucovorin Calcium Tablets, please consult complete prescribing information.

INDICATIONS AND USAGE: Leucovorin Calcium Tablets are indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (eg, methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS: *General.* Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia, a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions. Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin, and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy. Teratogenic Effects. Pregnancy Category C. Animal reproduction studies have not been conducted with leucovorin. It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Leucovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother.

Pediatric Use. (See Drug Interactions.)

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for 72 hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the premethotrexate serum creatinine, the leucovorin dosage should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[7,8] The recommended dosage of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (ie, trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[9,10,11]

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REVIEW

Immunologic Classification of Leukemia and Lymphoma

By Kenneth A. Foon and Robert F. Todd, III

Important insights into leukocyte differentiation and the cellular origins of leukemia and lymphoma have been gained through the use of monoclonal antibodies that define cell surface antigens and molecular probes that identify immunoglobulin and T cell receptor genes. Results of these studies have been combined with markers such as surface membrane and cytoplasmic immunoglobulin on B lymphocytes, sheep erythrocyte receptors on T lymphocytes, and cytochemical stains. Using all of the above markers, it is now clear that acute lymphoblastic leukemia (ALL) is heterogeneous. Furthermore, monoclonal antibodies that identify B cells, such as the anti-B1 and anti-B4 antibodies in combination with studies of immunoglobulin gene rearrangement, have demonstrated that virtually all cases of non-T-ALL are malignancies of B cell origin. At least six distinct subgroups of non-T-ALL can now be identified. T-ALL is subdivided by the anti-Leu-9, anti-Leu-1, and antibodies that separate T lymphocyte subsets into three primary subgroups. Monoclonal antibodies are also useful in the subclassification of non-Hodgkin's lymphoma, and certain distinct markers can be correlated with morphologic classification. The cellular origin of the malignant Reed-Sternberg cell in Hodgkin's disease remains uncertain. A substantial number of investigators favor a myelocyte/macrophage origin based on cytochemical staining; however, consistent reactivity with antimonocyte reagents has not been demonstrated. Although monoclonal antibodies are useful in distinguishing acute myeloid from acute lymphoid leukemias, they have less certain utility in the subclassification of acute myelogenous leukemia (AML). Attempts to subclassify AML by differentiation-associated antigens rather than by the French-American-British (FAB) classification are underway in order to document the potential prognostic utility of surface markers. Therapeutic trials using monoclonal antibodies in leukemia and lymphoma have been reported. Intravenous (IV) infusion of unlabeled antibodies is the most widely used method; transient responses have been demonstrated. Antibodies conjugated to radionuclides have been quite successful in localizing tumors of <1 cm in some studies. Therapy trials with antibodies conjugated to isotopes, toxins, and drugs are currently planned. Purging of autologous bone marrow with monoclonal antibodies and complement in vitro has been used in ALL and non-Hodgkin's lymphoma; preliminary data suggest that this approach may be an effective therapy and may circumvent many of the obstacles and toxicities associated with in vivo monoclonal antibody infusion.

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RECENT ADVANCES in immunology have led to important insights into leukocyte differentiation and the cellular origin of leukemia. It is now possible to define stages of human lymphocyte and granulocyte differentiation precisely using highly specific monoclonal antibodies that define cell surface antigens and molecular probes that identify rearrangement of immunoglobulin and T cell receptor genes. These can be combined with more traditional cell markers such as surface membrane (SmIg) and cytoplasmic immunoglobulin (CIg) on B lymphocytes, sheep erythrocyte receptors on T lymphocytes, and cytochemical stains. In this review, we summarize advances in the classification of leukemia and lymphoma and their importance in our understanding of normal leukocyte differentiation and therapeutic implications.

CELL MARKERS

B lymphocytes. B lymphocytes are usually identified by the presence of SmIg. Progenitors of B lymphocytes, commonly referred to as "pre-B cells," are present in fetal liver and normal bone marrow; the cells display cytoplasmic μ-heavy chain ($C\mu$) but lack intracytoplasmic light chain and SmIg. B and pre-B lymphocytes may also have receptors for the third component of complement (C'3) and for the Fc portion of IgG. Fc and C'3 receptors are not specific for the B cell lineage and are found in other cells such as monocytes and some nonhematopoietic cells. Similarly, histocompatibility-related antigens (Ia or HLA-DR) are also found on the surface of B cells, but are not unique to them.[1-4] Plasma cells are the most mature B lymphocytes; they lack detectable

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Table 1. Monoclonal Antibodies Reactive with Human B Lymphocytes

Antibody (Subclass)	Pattern of Reactivity	Mol wt of Antigen (kd)	Cluster Designation	Reference
BA-1 (IgM)	B lymphocytes, granulocytes malignant B cells	45,55,65	CD24	4,5
FMC1 (IgM)	B lymphocytes, malignant B cells	NR	NA	6
FMC7	$<$ 50% B lymphocytes, some malignant B cells	NR	NA	7
Anti-B1 (IgG$_2$)	B lymphocytes, malignant B cells	35	CD20	8
Anti-B2 (IgM)	B lymphocytes, malignant B cells (receptor for Epstein-Barr virus and C3d)	140	CD21	9,10
Anti-B4 (IgG$_1$)	B lymphocytes, malignant B cells	40	CD19	11
Anti-B5 (IgM)	Activated B lymphocytes, malignant B cells	75	NA	12
P1153/3	B lymphocytes, malignant B cells	NR	NA	13
OKB1 (IgG$_1$)	B lymphocytes, malignant B cells	168	NA	14,16
OKB2 (IgG$_1$)	B lymphocytes, granulocytes	NR	NA	14,15
OKB4 (IgM)	B lymphocytes, malignant B cells	87	NA	14,15
OKB7 (IgG$_{2a}$)	B lymphocytes, malignant B cells	175	NA	14,15
Anti-HLB-1 (IgG$_{2a}$)	B lymphocytes, malignant B cells	NR	NA	16
41H.16 (IgG$_{2a}$)	B lymphocytes, malignant B cells	39	NA	17
Anti-BL1 (IgG$_1$)	Subpopulation of B lymphocytes, malignant B cells, granulocytes	Glycolipid	NA	15
Anti-BL2 (IgG$_{2a}$)	B lymphocytes, malignant B cells, activated T cells	68	NA	15,19
Anti-BL3 (IgG$_1$)	Subpopulations of B lymphocytes, some malignant B cells, plasma cells, activated T cells	105	NA	15
Anti-PCA-1 (IgG$_{2a}$) and anti-PCA-2 (IgG$_1$)	Plasma cells, malignant plasma cells, weakly on monocytes and granulocytes	NR	NA	20
Anti-PC-1 (IgM)	Plasma cells, malignant plasma cells	28	NA	21
LN-1 (IgM)	B lymphocytes, malignant B cells, epithelial tumors	Sialoantigen	NA	22
LN-2 (IgG$_1$)	B lymphocytes, malignant B cells (nuclear membrane and cytoplasm)	35	NA	22
HD6, HD39, 29-110 SJ10-1H11, SHCL-1	75% of B lymphocytes, most malignant B cells	135	CD22	23
MNM6, PL-13, Blast-2	Germinal center B cells, not on resting B cells, some malignant B cells	45	CD23	23

NR, not reported, NA, not applicable.

The anti-B series, anti PC-1, and anti PCA-1 are available through Coulter Immunology, Hialeah, Fla; BA-1 through Hybritech Inc, San Diego; and the OKB series through Ortho System, Inc, Raritan NJ.

Table 2. Monoclonal Antibodies Reactive with Human T Lymphocytes

Antibody	Pattern of Reactivity	Mol wt of Antigen (kd)	Cluster Designation	Reference
OKT1, anti-T1, anti-Leu-1, 10.2 (Lyt-2), SC-1, A50, T101	Pan-T lymphocyte, pan-thymocyte	65	CD5	26–34
OKT3, anti-T3, anti-Leu-4, UCHT1	Pan-T lymphocyte (mitogenic)	20,20,25	CD3	27,28,35,36
Anti-Ti	Anti-clonotypic (T cell antigen receptor)	49–51 (α) 43 (β)	NA	37–39
12.1, T411	Pan-T, subpopulation of B	120	CD6	40,41
OKT11, anti-T11, anti-Leu-5, 9.6 (Lyt-3)	Pan-T lymphocyte (sheep erythrocyte receptor)	40–50	CD2	42–44
3A1, anti-Leu-9 (4H9), WT1, 4A	Pan-T lymphocyte	40	CD7	45–48
OKT4, anti-T4, anti-Leu-3	T helper/inducer	55	CD4	26–28,49–52
Anti-TQ1	Subset of T inducer cells	NR	NA	53
OKT5, OKT8, anti-T8, anti-Leu-2	T cytotoxic/suppressor	32–43	CD8	26,27,51,52,54,55
OKT6, NA1/34, anti-Leu-6	Thymocytes	45	CD1	27,28,56
OKT9, 5E9	Thymocytes, lymphoblasts, monocytes (anti-transferrin)	90	NA	27,28,57
OKT10	Thymocytes	45	NA	27
Anti-Ta$_1$	Activated T lymphocytes	105	NA	58
Anti-Tac	Interleukin-2 receptor	55	CD25	59–61

NR, not reported, NA, not applicable.

The OKT series of antibodies is available through Ortho Systems, Inc, Raritan, NJ; Leu series through Becton Dickinson Co, Mountainview, Calif; anti-T through Coulter Immunology, Hialeah, Fla; Lyt through New England Nuclear, Boston; and T101 through Hybritech Inc, San Diego.

SmIg but have CIg. Unlike the CIg found in pre-B lymphocytes, CIg in plasma cells includes both heavy and light chains.

A number of heteroantisera and, more recently, monoclonal anitbodies that identify B cell-associated antigens have been described (Table 1).[4-22] Where applicable, the nomenclature and clusters of differentiation (CD) defined by the Second International Workshop on Human Leukocyte Differentiation Antigens are shown.[23-25]

T lymphocytes. T lymphocytes were initially identified by their ability to bind sheep erythrocytes spontaneously. T lymphocytes also react with T cell-specific antisera and anti-T cell monoclonal antibodies, which may also be used to identify T lymphocytes, and have proven to be more sensitive and discriminatory (Table 2).[26-61] Many of these antibodies react with immature T cells; others react with more mature T cells. Some of these antibodies identify antigens found on all T cells, whereas others occur only on T cell subsets.

Myeloid cells. Monoclonal antibodies to cell suface markers on peripheral blood myeloid cells and their bone marrow progenitors have been extensively investigated.[62-125] Some of these monoclonal antibodies detect antigens expressed by either peripheral blood monocytes or neutrophils. Other reagents identify surface markers common to monocytes and neutrophils; monocytes, neutrophils, and large granular lymphoid cells (LGL); monocytes and platelets; or neutrophils and LGLs (Table 3).

The expression of several monoclonal antibody-defined myeloid antigens corresponds to pathways of normal differentiation within the myeloid lineage. These antibodies are

Table 3. Representative Murine Monoclonal Antibodies That Identify Human Myeloid Cell Surface Antigens: Distribution of Antigen Expression Among Peripheral Blood Cells

Monocytes	Neutrophils	Neutrophils and Monocytes	Neutrophils, Monocytes, and Large Granular Lymphocytes	Monocytes and Platelets	Neutrophils and Large Granular Lymphocytes
Mo2 (CDw14,55)[62-64]*	B40.9[77]	MY7 (CDw13,160)[69]	Mo1 (CD11,155,94)†[62-64]	Mo4 (100)[63]	B73.1 (60-70)‡[112]
Mo3[63]	R1B19 (145,105)[77]	MY8[69]	OKM1 (CD11,155,94)†[106,107]	20.3 (CDw14)[104]	VEP13 (CD16)[113]
UC45 (45)[65]	82H5 (CD15)†[83]	Mo5 (CD11,94)[95]	OKM9 (CD11,155,94)†[108]	OKM5 (88)[108,110]	
UCHM1 (CDw14)[66]	80H.5[84]	B13.4[77,96]	OKM10 (CD11,155,94)†[108]	5F1 (CDw14,85)[98,103]	
UCHALF[66]	TG-1 (CDw15)[85]	B9.8[77,96]	B43.4[77,96]	MPA (135,93)[111]	
S16-144[67,68]	VIM-D5 (CD15,145,105)§[86]	B34.3[77]	αS-HCL 3 or anti-Leu M5 (CDw14, 150, 95)[109]	SmO[66]	
MY3 (55)[69]	FMC 10 (CD15)§[87]	AML-2-23[91]			
MY4 (CDw14)[69]	FMC 12 (CD15)§[87]	PM-81[97]			
MY9[70]	FMC 13 (CDw15)[87]	1G10 (CD15)§[98]			
D5D6[71]	AHN-1 (145,105)§[88,89]	M206 (180)[99]			
C10H5[71]	MY-1§[90]	MMA or anti-Leu-M1[100]			
63D3 or antimonocyte .1 (200)[72,73]	PMN6[91]	S4-7 (150)[67,68]			
	PMN29[91]	AHN-7‖[101]			
61D3 or antimonocyte .2 (75)[74]	PMN 7C3¶[92]	80H.1[84]			
	3G8 (60-70)‡[93]	80H.3 (CDw15)[84]			
	1B5[94]				
	4D1 (59)[94]				
MOP-15 (CDw14)[75]		DUHL60.1 (CDw15)[102]			
MOP-9 or anti-Leu-M3 (CDw14)[75]		DUHL60.3 (CDw15)[102]			
Mac-120 or anti-Leu-M2(120)[76]		DUHL60.4 (CDw13)[102]			
		L4F3[103]			
B44.1 (55)[77]		T5A7 (CDw17)[103]			
1D5[78]		20.2 (CDw12)[104]			
PHM3 (50)[79]		VIM-2[105]			
4F2 (40,80)[80,81]					
FMC17 (CDw14)[82]					

*Antibody/antigen (cluster designation [CD], antigen mol wt, kd, reducing conditions), key references (superscript).

†Anti-C3bi receptor antibodies.

‡Anti-Fc receptor antibody.

§Antibodies bind to X-hapten, lacto-*N*-fucose-pentaosyl III.

‖Antibody immunoprecipitates broad band of 28 to 65 kd on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

¶Antibody immunoprecipitates two broad bands of 155 to 288 and 75 to 125 kd on SDS-PAGE.

Table 4. Representative Murine Monoclonal Antibodies That Identify Human Myeloid Differentiation Antigens

	S16-114[67,68]	MY9[70,114]	82H5[83]	MY10[115,116]	L1B2[103,117]	R1B19[67,68,77,89]	80H.5[84]	S4-7[67,68]	AHN-7[101]	L4F3[103,117]	T5A7[103,117]	Mo1[95,114,118]	Mo5[95]	MY7[114,118,120]	1G10[98,103,117]	PM81[97,114]	AML-2-23[91,114,121,122]	MY-1[90,116,123]	TG-1[66,85]	PMN 6[91,122]	MY8[69,118,119]	80H.3[84]	B13.4[77,86]	B34.4[77]	Mo2[63,114,124]	MY3/4[114,118,119]	20.3[104]	Mo4[63,124]	5F1[98,103]	SFL 23.6[125]
CFU-GEMM	+	+	+			−		−				−				−	−								−	−			−	−
CFU-GM (d 14)	+	+	+	+	+	−	+	+	+	+	−	−		+	+	+	−	−	−	−	−				−	−			−	−
CFU-GM (d 7)		+			+	+		+		+	+	−		+	+	+	+		−		−				−	−			−	−
Myeloblast	+	+		−	+	+	+	+	+	+	−	−	−	+	+	+	+	+		−	−	−	−	−	−	−	+	−	−	−
Promyelocyte	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−
Myelocyte	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Metamyelocyte	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
Neutrophil	−	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Monoblast/ promonocyte		+		−	+	−	−		−	+	+	+	+	+	−	+	+		−	−	+	+	+	+	+	+	+	+	+	−
Monocyte	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	−
BFU-E	+	+	+	−	−	−	−	−		+	−	−		−	−	−	−		−	−	−					−			−	−
CFU-E	−	−			−	−	−	−		−	−	−		−	−				−		−					−			+	+
Erythroid precursor	−	−		−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−		−	−	−		+	−	+	+
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CFU-Mega			+							+																				
Megakaryocyte			−		−					−	−	−			−						−				−	−	+	+		
Platelet		−	−	−	−	−	−			−	−	−	−	−	−	−	−	−	−	−	−		−	−	−	−	+	+	+	−

therefore useful as tools for identifying hematopoietic cells at various stages of maturation and proliferative potential (Table 4 and Fig 1). Using either complement-dependent monoclonal antibody-mediated lysis (negative selection), or techniques such as fluorescence-activated cell sorting or immune rosetting (positive selection), it is possible to determine patterns of antigen expression by multipotent stem cells (CFU-GEMM) and by stem cells committed to the myeloid (CFU-GM), erythroid (BFU-E, CFU-E), or megakaryocyte/platelet (CFU-Mega) pathways of differentiation. In several reports, myeloid progenitor cells have been purified 50- to 100-fold from bone marrow mononuclear cells[85,126] enabling studies of morphologic and functional characteristics. Certain determinants are uniquely expressed by progenitor cells (eg, MY-10); other antigens are detectable on myeloid, erythroid, or platelet precursors corresponding to morphologically and histochemically distinct stages of maturation within the bone marrow. In the case of neutrophil differentiation, some antigens are either lost (Ia, MY-10) or acquired (Mo1, MY8, 80H.3, or B34.3) as cells progress from myeloblasts to mature neutrophils. Expression of other determinants (82H5, R1B19, S4-7) are maintained on all recognizable myeloid cells.

Although many of these antigenic determinants operationally serve as differentiation markers, some represent epitopes on functionally significant plasma membrane proteins, glycoproteins, and glycolipids. The glycoprotein heterodimer identified by the murine monoclonal antibodies Mo1 and OKM1 is a receptor for the binding of particles opsonized with C3 cleavage product C3bi (CR3 activity).[127,128] Antibodies B73.1 and 3G8 identify an Fc receptor for IgG that is expressed by LGL and/or neutrophils.[93,112] Data from immu-

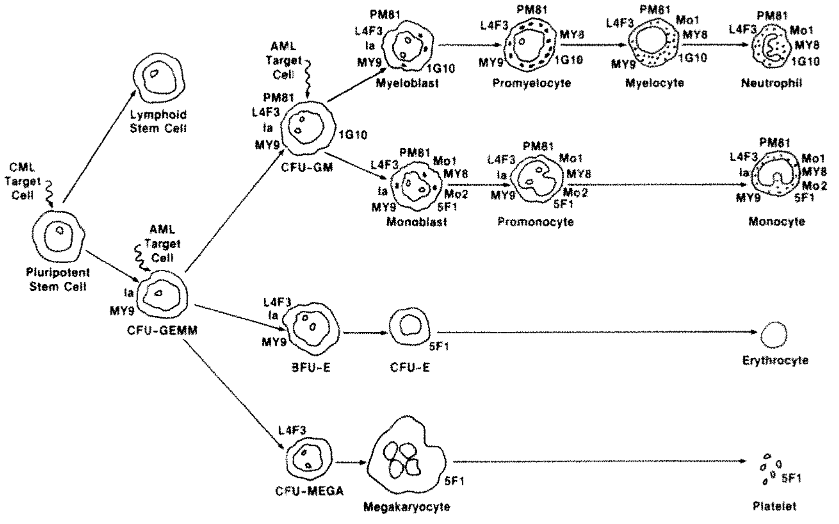

Fig 1. Schematic representation of human myeloid differentiation indicating the cell surface marker phenotype (as defined by selected well-characterized monoclonal reagents) of identifiable maturational steps. Phenotypic expression by monoblasts and promonocytes is tentative and based on positive expression by immature-appearing monocytoid cells in bone marrow. The phenotypes of CFU-GEMM, CFU-GM (early *or* late), BFU-E, CFU-E, and CFU-MEGA are based on the outcome of positive and/or negative selection (antibody-dependent, complement-mediated lysis, immunorosetting, or cell sorting) of human bone marrow cells on subsequent colony growth. Phenotypes of other cells are based on immunofluorescence analyses. Hypothesized sites of CML and AML leukemogenesis are indicated.

Table 5. Monoclonal Antibodies Leukemia-Associated Antigens

Antibody (Subclass)	Pattern of Reactivity	Molecular wt of Antigen (kd)	Cluster Designation	Reference
J5, BA-3, anti-CALLA	Most non-T-ALL, Burkitt's lymphoma, follicular lymphoma, some lymphoblastic lymphoma and T-ALL, rare normal cells	100	CD10	130-136
RFB-1	Myeloid progenitor cells, immature lymphoid bone marrow cells	NR	NA	141
BA-2, SJ-9A4 Du-ALL-1	Lymphohematopoietic bone marrow progenitor cells, most non-T-ALL, platelets	24	CD9	137-140, 142
Anti-3-3	T-ALL	35-40	NA	143
Anti-3-40	T-ALL, some non-T-ALL, rare AML vinentin and keratin intermediate filaments in normal cells	35-40	NA	143, 144
SN1	T-ALL	NR	NA	145
CALL2	T-ALL	NR	NA	146

NR, not reported; NA, not applicable.

J5 is available through Coulter Immunology, Hialeah, Fla; BA-2 and BA-3 through Hybritech Inc, San Diego.

noprecipitation or immunoblotting analyses indicate that several of these antibodies are specific for epitopes on the same parent structure or even the same epitope.

Leukemia-associated antigens. The common acute lymphoblastic leukemia-associated antigen (CALLA) was originally defined by antiserum produced in rabbits by immunization with SmIg-negative, sheep erythrocyte rosette–negative acute lymphoblastic leukemia (ALL) cells.[129] This antiserum reacted with a 100 kd glycoprotein antigen. Monoclonal antibodies that recognize CALLA (CD10) have recently been described (Table 5).[130,131] Although CALLA is absent on normal peripheral blood lymphocytes, it is not leukemia specific, and is present on normal terminal deoxynucleotidyl transferase (TdT) and Ia antigen–positive bone marrow cells that are thought to be lymphohematopoietic precursor cells.[132] CALLA has also been identified on renal tubular and glomerular cells, mammary epithelium, fetal small intestine epithelial cells,[133] granulocytes,[134] fibroblasts,[134] and melanoma cell lines.[135] CALLA is also present on Burkitt lymphoma cells, follicular lymphoma cells, and cells from 40% of patients with lymphoblastic lymphoma.[136]

P24/BA-2 is a second leukemia-associated antigen with a mol wt of 24 kd defined by the BA-2,[137] SJ-9A4,[138] and DU-ALL-1[139] monoclonal antibodies (CD9). These antibodies do not react with any normal circulating hematopoietic cells except for platelets. They react with most non-T ALL cells and, like CALLA, the P24/BA-2 antigen is present on ~3% to 9% of sheep erythrocyte receptor and SmIg negative TdT positive bone marrow mononuclear cells. This may also represent a bone marrow-derived lymphoid progenitor cell. The anti-J2 antibody[140] has a similar pattern of reactivity as do the BA-2, SJ-9A4, and DU-ALL-1 antibodies. The J2 antigen is also present on activated normal T lymphocytes and may react with the same surface molecule as BA-2. The RFB-1 antibody likewise reacts with immature lymphoid cells in the bone marrow,[141] but differs from BA-2 in its reactivity with myeloid progenitor cells.[141,142] The anti-3-3, anti-3-40,[143,144] SN1,[145] and CALL2[146] antibodies identify antigens found on T-ALL cells but not generally on non-T-ALL or other malignant or normal hematopoietic cells. A summary of the most frequently referenced antibodies and their cluster designations is presented in Table 6.

Immunoglobulin and T cell receptor genes. Recombinant DNA technology has provided important insights into antibody diversity and antigen-specific T cell receptors.[147,148] Immunoglobulins are composed of heavy and kappa and lambda light chains, encoded by genes on chromosomes 14, 2, or 22, respectively.[149-152] Immunoglobulin genes are encoded by discontinuous segments of DNA.[153-158] At one point in development, a potential antibody-producing cell must productively rearrange variable, diversity, and joining genes

Table 6. Frequently Referenced Antibodies with Cluster Designations

Cluster Designation	Antibody
CD1	OKT6, anti-Leu-6, NA1/34
CD2	OKT11, anti-T11, anti-Leu-5, 9.6
CD3	OKT3, anti-T3, anti-Leu-4, UCHT-1
CD4	OKT4, anti-T4, anti-Leu-3
CD5	OKT1, anti-T1, anti-Leu-1, 10.2, T101
CD6	12.1, T411
CD7	anti-Leu-9, 3A1, WT1, 4A
CD8	OKT5, OKT8, anti-T8, anti-Leu-2
CD9	BA-2, SJ-9A4, Du-ALL-1
CD10	J5, BA-3, anti-CALLA
CD11	Mo1/OKM1, Mo5
CDw12	20.2
CDw13	DUHL60.4, MY7
CDw14	Mo2, MY4, MOP-15, FMC 17
CD15	FMC10, VIM-D5, DUHL60.1
CD16	VEP13
CDw17	T5A7
CD19	anti-B4
CD20	anti-B1
CD21	anti-B2
CD22	SHCL-1, HD6, HD39, 29-110
CD23	PL13, MNM6, Blast-2
CD24	BA-1
CD25	anti-Tac

(VDJ), which are then linked to the constant region locus. Immunoglobulin gene rearrangements are hierarchical; μ heavy chain rearrangements precede light chain rearrangements, and κ light chain rearrangement precedes λ light chain rearrangements.[156–158] These rearrangements can be detected by Southern blot analyses of DNA from B cells using appropriately radiolabeled heavy or light chain probes. Heavy chain rearrangements have been identified in non-B cells, but light chain rearrangements appear to be restricted to B cells.[157,159,160] Clonal rearrangements of light chain genes are therefore an extremely sensitive tool to identify B cell malignancies.

The antigen-specific T cell receptor is a heterodimer formed by a 40- to 50-kd α subunit ($T\alpha$), and a 40- to 45-kd β subunit ($T\beta$).[161] It is associated with three 20- to 25-kd peptide chains identified by the T3 monoclonal antibody.[161] Recently, cDNA clones to the $T\beta$ and $T\alpha$ receptors have been isolated.[162–166] The human $T\beta$ receptor gene has been localized to chromosome 7[167] and the human $T\alpha$ receptor gene maps to chromosome 14.[168] The less well-defined $T\gamma$ receptor gene is currently under investigation. The T cell receptor genes undergo rearrangements in a fashion analogous to that of immunoglobulin genes. The $T\gamma$ receptor gene has been shown to be rearranged in murine cytotoxic T lymphocyte cell lines but not myeloma cell lines.[169] Similar to the $T\beta$ chain gene, the $T\gamma$ gene rearrangement appears to occur early in T cell development, whereas $T\alpha$ chain expression occurs later in thymic ontogeny.[170]

T_β gene rearrangements have been detected in malignant human T cells by Southern blotting.[171–173] This technique can detect as few as 1% tumor cells in a mixed cell population[171]; it is a sensitive diagnostic marker for T cell diseases. Rearrangements of the $T\beta$ antigen receptor are reported in 25% of patients with non-T ALL,[174] and in a small proportion of patients with B cell leukemia.[175] This is similar to the rearrangement of immunoglobulin heavy chain genes in 10% of patients with T cell leukemia.[159,160]

Intracellular enzymes and biochemical markers. TdT is present in thymocytes and in a small percentage of bone marrow cells, but not in mature lymphocytes.[176,177] TdT is identified in all subtypes of ALL and is, therefore, not discriminative. TdT has also been demonstrated in a small proportion of acute myelogenous leukemia (AML) cells.[178] Other intracellular enzymes reported useful in identifying subsets of ALL include hexosaminidase, adenosine deaminase,[180] 5'-nucleotidase,[181] purine nucleoside phosphorylase,[182,183] and acid phosphatase.[184] Acid phosphatase is present in T-ALL cells but not in non-T-ALL cells. Cytochemical reactions are useful in the subclassification of AML.[185,186] The M1 through M3 myeloid subtypes contain myeloperoxidase and sometimes nonspecific esterase. Myelomonocytic leukemia cells (M4) also contain myeloperoxidase and nonspecific esterase; the latter is variably inhibited by sodium fluoride. Acute monocytic leukemia (M5) cells contain myeloperoxidase and nonspecific esterase which is completely inhibited by sodium fluoride.

Several surface membrane-associated biochemical markers of leukemia cells have also been described. The glycolipid asialo GM1 is found on cells from patients with ALL (non-T-ALL and T-ALL) but not on cells from patients with other forms of leukemia.[187] Alterations in membrane carbohydrates, such as decreased complex gangliosides,[188] carbohydrate-containing antigens,[189] and receptors for cholera toxin[190] have been reported on leukemia cells.

CLASSIFICATION OF THE LYMPHOID LEUKEMIAS AND LYMPHOMAS

Acute lymphoblastic leukemia. ALL is heterogeneous. The first surface markers used to differentiate subclasses of ALL were receptors for sheep erythrocytes,[191–193] which identify a T cell subset (15% to 20% of cases), and SmIg, which identifies a B cell subset (<5% of cases). Both T and B cell subgroups have an unfavorable prognosis.[194,195] The next important advance in identifying ALL was the development of an antiserum to CALLA.[129] CALLA reactivity identified a non-B, non-T subclass of ALL patients (~70% of cases) with a more favorable prognosis than T-ALL, B-ALL, or non-B, non-T-ALL without CALLA.[195] Other markers such as Ia antigen were commonly found on non-T-ALL and could help differentiate non-T-ALL from T-ALL.[196] By testing for $C\mu$ heavy chain, a subset designated pre-B ALL has been identified.[197–199] Except for the presence of $C\mu$, this subset expresses the same surface markers as the CALLA form of non-T-ALL; it appears, however, to have a less favorable prognosis.[200]

With the development of monoclonal antibodies, it became evident that the T cell subset of ALL was heterogeneous.[201–205] More recently, studies employing immunoglobulin gene rearrangements and monoclonal antibodies that identify B cell-associated antigens have demostrated that most cases of non-T-ALL are derived from the B cell lineage.[156,206–208] We review these data and present a new classification for ALL based on these recent observations.

Non-T-ALL. Two important areas of research have prompted a reassessment of non-T-ALL. First, monoclonal antibodies that recognize B cell-associated antigens have been identified; many are present on non-T-ALL cells. The most specific of these antibodies is probably anti-B4, which react with 95% of cases of non-T-ALL.[11,208] Second, clonal rearrangements of immunoglobulin genes provide strong evidence for the B cell lineage of most cases of non-T-ALL.[156,206,208]

Although Ia antigen is present on most non-T-ALL cells, and CALLA is present in 75% of cases of non-T-ALL, these antigens are also identified on ~10% of cases of T-ALL. Therefore, B cell-associated antigens (Table 1), which are not identified on T-ALL cells, are the most useful in distinguishing non-T-ALL. The B1 and B4 antigens are model antigens for this discussion.

Less than 5% of cases of ALL express SmIg (usually IgM); these cells are typically classified as B-ALL. These cells generally express other B cell antigens, including B1 (CD20), B4 (CD19), and Ia. B-ALL in children is probably a leukemic phase of non-Hodgkin's or Burkitt's lymphoma.[193,194] Another marker that identifies a subset of non-T-ALL is $C\mu$ heavy chain; κ and λ light chains and SmIg are typically absent.[197] These cells are considered pre-B cells. As indicated, most cases of non-T-ALL cells of B lineage; thus Cμ is useful in determining the level of differentiation. Pre-B cells that synthesize μ heavy chain are more mature than those pre-B cells that do not synthesize μ heavy chain, but are less mature than those with SmIg.

Nadler and co-workers[208] recently classified 138 patients with non-T-ALL. They divided these cases into four major subgroups. The first subgroup was Ia antigen positive, representing 5% of cases. Another subgroup expressed the Ia and B4 antigens, representing 15% of cases. The third subgroup expressed the Ia, B4, and CALLA antigens, comprising one third of the cases. Finally, one half of the cases of non-T-ALL were Ia, B4, CALLA, and B1 positive. The fourth group was further subdivided into cases with and without Cμ. We propose that cases with Cμ be placed in a separate group (group V). The final and most differentiated group, group VI, represents SmIg-positive B-ALL (Table 7).

Nadler and co-workers[208] also studied immunoglobulin gene rearrangements in cells from patients in groups II, III, and IV. Patients in group II (Ia and B4 positive) demonstrated rearranged heavy chain genes, with germ lines for both κ and λ light chain genes. Based on these data, they hypothesized that the group II phenotype represents the earliest stage in B cell maturation. Patients in group III had rearranged heavy chain genes and most had κ light chain gene recombination, either rearrangement or deletion. Patients in group IV had rearranged heavy chain genes; two of four had deletions of κ.

Virtually all non-T ALL cells have immunoglobulin heavy chain rearrangement; not all of them, however, demonstrate light chain rearrangements. Because heavy chain rearrangements also occur in non-B cells[159,160] immunoglobulin heavy chain rearrangements are insufficient to assign non-T-ALL to the B cell lineage. Nadler and co-workers have proposed that the B4 antigen provides the most important independent parameter with which to identify B cell-derived non-T cell ALL. More recently, up to 25% of non-T ALL have been reported to have rearrangements of the Tβ receptor.[174] This observation emphasizes the necessity of combining DNA genotyping and surface marker analyses for more precise classification of leukemia and lymphoma. The remaining 5% of morphologically and cytochemically defined non-T cell ALLs (group I), which express the Ia but not the B4 antigen, most likely represents the earliest stage of pre-B cell differentiation. This hypothetical scheme of early B cell differentiation is further supported by data demonstrating that all of the proposed ALL subgroups can be identified in normal fetal liver and bone marrow and in normal adult bone marrow.[208]

Table 7. Classification of Non-T-ALL

Group	Antigens				Cytoplasmic μ	Surface Membrane Immunoglobulin
	Ia	B4	CALLA	B1		
I	+	−	−	−	−	−
II	+	+	−	−	−	−
III	+	+	+	−	−	−
IV	+	+	+	+	−	−
V	+	+	+	+	+	−
VI	+	+	+/−	+	−	+

T-ALL. T-ALL represents 15% to 25% of cases of ALL. Clinical features associated with T-ALL include a high blast cell count, predominance of older male patients, and mediastinal masses. T-ALL was originally identified by rosetting with sheep erythrocytes. The most sensitive marker for T-ALL is probably the pan-T 40 kd antigen identified by the anti-Leu-9 antibody (CD7). This antigen is present on most thymocytes and T cells but not on non-T-ALL or B cell lymphomas or leukemias.[45-48] In a study of 23 patients with T-ALL, all cases expressed the Leu-9 antigen.[46] Although it was previously reported that anti-Leu-1 (CD5) is the most sensitive marker for T-ALL,[202,209] three of these 23 patients whose cells were anti-Leu-9 positive were negative with anti-Leu-1.[46] Anti-Leu-9 reacts with a small proportion of cases that appear to be myeloid leukemias.[210] In addition, an unusually high incidence of CALLA, Ia, and BA-2 expression has been reported in adults with T-ALL.[211] Recently, rearrangement of the Tβ receptor gene in cases of T-ALL has been reported.[171-175] Two cases of T-ALL with the phenotype of early thymocytes were reported to have no rearranged Tβ receptor genes, suggesting that Tβ rearrangement may occur later in thymocyte development.[212]

Further subclassification of T-ALL is controversial. Reinherz and colleagues proposed a subclassification for T-ALL according to the level of thymic differentiation.[201] Several elements of their subclassification of T-ALL have been confirmed; others are controversial. The most primitive thymocytes, referred to as early or stage I thymocytes, react with T9 and T10 antibodies and account for ~10% of the thymic cells. In their study, Reinherz and co-workers reported that most T-ALL cells express antigens found on early thymocytes. The next level of thymic differentiation, which includes the majority of thymocytes, is referred to as common or stage II. These cells lose T9, retain T10, and acquire T6 (CD1), T4/Leu-3 (CD4), and T8/Leu-2 (CD8) antigens. Approximately 20% of cases of T-ALL express this phenotype. Mature stage III thymocytes no longer express T6 but segregate into T4/Leu-3 or T8/Leu-2 subsets similar to peripheral blood T lymphocytes. Only rarely did Reinherz and colleagues find T-ALL cells with the phenotype of mature thymocytes or circulating T lymphocytes. In a more recent study, Roper and coworkers[204] confirmed many of the findings reported by Reinherz and colleagues, but reported some major differences. In this study, only one third of the T-ALL patients had the phenotype of early or stage I thymocytes; most had the phenotype of either intermediate or late stage thymocytes.

In Table 8, we summarize these data and propose a scheme for the classification of T-ALL. The common marker for all of the subgroups is Leu-9. Nearly all cells also express Leu-1 and most express T11/Leu-5 (CD2) that identifies the receptor for sheep erythrocytes. Cells in subgroup I also express T9 and/or T10. The pan T antigen, identified by T3/Leu-4 (CD3), represents a mature antigen and is not found on group I cells. The T4/Leu-3 helper-associated antigen, the T8/Leu-2 suppressor-associated antigen, and the T6 antigen are not expressed on group I cells.

The next level of differentiation is group II. T9 is found on some cells;[204] however, the T6 antigen as well as the simulta-

Table 8. Classification of T-ALL

	Antigens						
Group	Leu-9*	Leu-1	T11/Leu-5	T3/Leu-4	T4/Leu-3	T8/Leu-2	T6
I	+	+ (90%)	+ (75%)	−	−	−	−
II	+	+	+	+ (25%)	+ (90%)	+ (90%)	+
III	+	+	+	+	+/−†	+/−†	−

*Found on virtually all T-ALL cells.

†No longer simultaneous expression of T4/Leu-3 and T8/Leu-2 as found in group II.

neous expression of T4/Leu-3 and T8/Leu-2 antigens clearly distinguish group II from group I. Some cells in group II may also express T3/Leu-4. Group III T-ALL cells lose the T6 antigen and segregate into cells that have the phenotype of mature thymocytes and T lymphocytes (T3/Leu-4, T4/Leu-3 or T3/Leu-4, T8/Leu-2).

Although Roper and co-workers[204] searched for clinical correlations among these three groups of T-ALL, they found no unique clinical features among the subgroups and no differences in remission duration or survival. However, the groups were too small for statistically valid conclusions. Presently, we believe it useful to subclassify T-ALL using this system, so that data from a number of institutions can be analyzed for clinical correlations between the subgroups of T-ALL (Table 8).

Non-Hodgkin's lymphoma. The non-Hodgkin's lymphomas are a diverse group of neoplasms whose pathologic classification is controversial. It is even more difficult to correlate pathologic classification with immunologic classification. Several immunologic patterns emerge, however, and we will attempt to place them within the non-Hodgkin's lymphoma working classification[213] as well as the Rappaport classification.[214,215]

Follicular or nodular lymphomas. The follicular or nodular lymphomas most likely represent neoplastic proliferation of lymph node-derived follicular center B lymphocytes. The cell type may be a small cleaved cell (nodular lymphocytic poorly differentiated lymphoma by the Rappaport classification), mixed small cleaved and large cleaved or noncleaved cells (nodular mixed), or predominantly large cell (nodular histiocytic). The first two cell types fall within the working classification as low-grade lymphoma, whereas the latter cell type is classified as an intermediate-grade lymphoma. Although the predominantly small cleaved cell will almost always express high-density monoclonal SmIg, larger cells may be SmIg negative.[216,217] However, the small cleaved and large cells will routinely express Ia, B4, and B1 antigens and will often express the B2 antigen.[217] More than half of these cases will also express CALLA.[217,218] Follicular lymphoma cells may be found in the peripheral blood as a "leukemic" phase of the disease (formerly referred to as lymphosarcoma cell leukemia). These cells can usually be differentiated from chronic lymphocytic leukemia (CLL) cells because they may express CALLA, which is not expressed on CLL cells; they do not express the T1/Leu-1 pan-T antigen found on CLL cells; and they generally will have a low percentage of mouse erythrocyte rosette formation (see below).[219,220]

Malignant lymphoma, small lymphocytic. Malignant lymphoma, small lymphocytic (diffuse lymphocytic well-differentiated lymphoma in the Rappaport classification) is a low-grade malignancy, and some cases may be identical to CLL. Also included within this subclassification are the plasmacytoid lymphocytic subgroups with and without an IgM monoclonal gammopathy; some of these cases are similar to Waldenström's macroglobulinemia (described below). Surface markers on these small lymphocytic cells include low-intensity SmIg, mouse erythrocyte receptors, C'3 and receptors for the Fc portion of IgG, and Ia, B1, B2, B4, BA1, and other B cell antigens. These features are similar to CLL, and the cells also express the T1/Leu-1 pan-T antigen.

Malignant lymphoma, diffuse small cleaved cell and diffuse mixed small and large cell. Malignant lymphoma, diffuse small cleaved cell (diffuse lymphocytic poorly differentiated lymphoma in the Rappaport classification) is an intermediate prognostic group. The cells are B lymphocytes that (similar to follicular lymphoma cells) usually display large amounts of monoclonal SmIg. Unlike follicular lymphoma cells, however, they do not usually express CALLA.[218] Similar to follicular lymphoma cells, they do not express the T1/Leu-1 antigen as do cells from most small lymphocytic lymphomas and CLL. However, all these cell types have in common the expression of Ia, B4, B1, B2, and other B cell antigens.[217]

The diffuse mixed small and large cell (diffuse mixed lymphocytic-histiocytic) lymphomas have not been extensively studied but are most likely predominantly B cell diseases. They are also considered an intermediate-grade prognostic group.

Malignant lymphoma, diffuse large cell and large cell immunoblastic. In the working classification, the diffuse large cell lymphomas are considered within the intermediate prognostic group, whereas large cell immunoblastic lymphoma is a high-grade malignancy. By the Rappaport classification, both of these cell types would be described as histiocytic. This is clearly a misdesignation since 80% to 90% of cases represent clonal expansions of malignant B cells.[217,221] A high percentage of these cells express T9 and T10 antigens.[216] Fifty-seven cases of diffuse large cell lymphoma were recently studied and divided into the following subgroups: (a) B1, B4, and SmIg positive; B2 negative (50%); (b) B1, B4, SmIg, and B2 positive (30%); (c) B1 and B4 positive; SmIg and B2 negative (10%); and (4) B1 and SmIg positive, and B2 negative (10%).[222] These data suggest that most of these lymphomas represent the malignant counterpart of B cells at the midstage of differentiation. Ten to 20 percent of cases are T cell lineage; 2% are derived from the monocyte-myeloid lineage. Recently, clonal rearrangement of the Tβ receptor has been described in patients with T-derived non-Hodgkin's lymphoma.[172,173]

Malignant lymphoma, lymphoblastic. Malignant lymphoma, lymphoblastic, or lymphoblastic lymphoma, is a high-grade malignancy. The nuclear membrane is characteristically deeply subdivided, exhibiting either a lobulated (convoluted) appearance or a fine linear (nonconvoluted) subdivision in a round nucleus. Lymphoblastic lymphoma represents approximately one-third of the cases of non-Hodgkin's lymphomas in children and 5% of cases in adults. The disease is more prevalent in males; these patients often have a mediastinal mass. In some cases, the disease may evolve into a leukemic phase morphologically indistinguishable from T-ALL. The malignant cells are T cells, form E rosettes, react with T cell antisera,[223-225] and have rearrangements of the Tβ receptor.[226] Studies with monoclonal antibodies have demonstrated marked heterogeneity. Lymphoblastic lymphoma cells differ from T-ALL in that the cells rarely express the surface markers common to immature thymocytes (group I);[227] phenotypes are equally divided between group II and group III T-ALL. In 40% of cases, the cells are reported to express CALLA; CALLA expression is less common in T-ALL (10%).[218]

Malignant lymphoma, small noncleaved cell. Malignant lymphoma, small noncleaved cell includes Burkitt's lymphoma and other lymphomas previously designated undifferentiated non-Burkitt type (high grade). Burkitt cells from peripheral blood and bone marrow are usually classified as L3 by the FAB criteria.[185,186] Most cases of Burkitt's lymphoma from Africa are endemic and are associated with the Epstein-Barr virus (EBV). Most non-African cases (nonendemic) are EBV negative.[228] Chromosomal abnormalities involving chromosome 8 (carrying the oncogene c-*myc*) and either 2, 14, or 22 occur in virtually all cases of endemic and nonendemic Burkitt's lymphoma.[229] These are designated t(2;8), t(8;14) and t(8;22), respectively. Usually the light chain class expressed on these cells is correlated with the translocation, ie, κ in t(2;8) and λ in t(8;22). African Burkitt's lymphoma cells have receptors for C'3 and for the Fc portion of IgG in addition to the EBV receptor. American Burkitt's lymphoma cells do not express these receptors.[228] Phenotyping of cell lines derived from patients with undifferentiated lymphoma of the Burkitt's and non-Burkitt's type have demonstrated heterogeneity.[230] These studies suggest that Burkitt cells follow a divergent pathway of B cell evolution because they are all TdT negative (unlike early B cell non-T-ALL). The most primitive of the Burkitt cell lines are Ia and B1 positive and may or may not express CALLA. Maturation was evident in other Burkitt cell lines by the expression of Cμ, surface membrane IgM, and/or IgM secretion. Some of these Burkitt cell lines also expressed the Tac antigen.

Peripheral T cell lymphoma. Peripheral T cell lymphoma would usually be classified as malignant lymphoma, large cell immunoblastic (high grade) under the working formulation. However, this tumor has unique features and will be described separately. The term "peripheral T cell lymphoma" is used to distinguish it from lymphoblastic lymphoma of presumed thymic origin. Peripheral T cell lymphomas are thought to derive from peripheral T lymphocytes in lymph nodes and other nonlymphoid sites. These lymphomas comprise a broad spectrum of morphologic types of lymphocytes. In all instances, the cells have T cell markers admixed with epithelioid histiocytes, plasma cells, eosinophils, and vascular hypertrophy. Clinically, peripheral T cell lymphoma is characterized by generalized lymphadenopathy, weight loss, and a high incidence of pulmonary involvement.[231] Surface markers are usually but not always characteristic of mature T helper cells,[232] including the T4/Leu-3 helper-associated antigen and the T3/Leu-4, T11/Leu-5, and T1/Leu-1 pan-T antigens. Rearrangement of the Tβ receptor has been reported.[226]

Tγ lymphoproliferative disease. Tγ lymphocytes are a subset of T lymphocytes with receptors for the Fc portion of IgG. A high proportion of normal Tγ lymphocytes are LGL. These cells are thought to be responsible for natural killer (NK) and antibody-dependent cell-mediated cytotoxicity in humans[233] and rodents.[234] A lymphoproliferative disorder made up of predominantly Tγ lymphocytes has been described; we refer to this as chronic Tγ lymphoproliferative disease.[235] Typically, patients are elderly males with increased Tγ lymphocytes infiltrating the bone marrow and spleen.[235,236] Although the disease is not rapidly progressive, neutropenia and recurrent infections are common. Most patients do not require chemotherapy. Variants of this disease, including a more aggressive form, have been described.[237] Clonal chromosomal abnormalities,[238] as well as clonal rearrangement of the Tβ receptor, have been reported.[175,239] Cells from chronic Tγ lymphoproliferative disease usually contain acid phosphatase and β-glucuronidase and express the pan-T antigens T3/Leu-4, T11/Leu-5, the suppressor-associated antigens T8/Leu-2, and the NK-associated antigen Leu-7 (HNK-1). Other monoclonal antibodies that react with LGL[240,241] may also prove to be useful.

Cutaneous T cell lymphoma (mycosis fungoides, Sézary cell leukemia). Skin lesions are the most prominent feature of patients with cutaneous T cell lymphoma.[242] Lesions vary from limited plaques to diffuse generalized plaques, tumors, and generalized erythroderma. Rare patients with limited plaque disease and <50% with generalized plaques and tumors have extracutaneous disease detected by light microscopy evaluation of peripheral blood and lymph nodes. Special studies including cytogenetic analysis and electron microscopy indicate blood involvement in >50% of patients with limited plaque disease and most patients with generalized plaques and skin tumors.[243] Analysis of the Tβ receptor rearrangement will likely reveal a higher proportion of cases with nonmalignant cells in the blood and lymph nodes.

The malignant cells in this disorder are characterized by a cerebriform nucleus. In the skin, the cells are referred to as mycosis fungoides cells and in the peripheral blood as Sézary cells. Sézary and mycosis cells form E rosettes, react with T antisera and anti-T monoclonal antibodies,[244,245] and have clonal rearrangements of the Tβ receptor.[173,175,226,246] In most cases, the cells express the phenotype associated with normal helper/inducer T lymphocytes (T-1/Leu-1, T3/Leu-4, T4/Leu-3 positive)[247-251] and function as helper T lymphocytes in in vitro assays.[252] The 3A1 antibody (CD7), which reacts with >85% of normal circulating T lymphocytes and with mycosis cells in the skin, does not generally react with Sézary cells in the blood.[253]

Adult T cell leukemia/lymphoma. Adult T cell leukemia/lymphoma is associated with a human retrovirus designated human T cell leukemia/lymphoma virus-1 (HTLV-1).[254,255] Virtually all patients tested have antibodies to HTLV-1.[256] Patients with this disease have been identified primarily in Japan, the United States, and the Caribbean. In the United States, the patients are young (median age 33 years), predominantly black, and born in the southeast.[253] Common clinical features include a rapid onset of symptoms with rapidly progressive cutaneous lesions and hypercalcemia. Skin lesions are variable and include small and large discrete or confluent nodules, or nonspecific plaques, papules, or patches. Patients have increased bony turnover with abnormal bone scans and elevated alkaline phosphatase and may have lytic bone lesions.[257] Lymphocytosis is common, and circulating malignant cells are present in low numbers in most patients. Peripheral lymphadenopathy is common, with retroperitoneal and hilar involvement in approximately 50% of cases. Bone marrow, gastrointestinal, pulmonary, leptomeningeal, and hepatic involvement are somewhat less common (20% to 50%). Response to combination chemotherapy is prompt and often complete, but duration of response is short (median 13 months). Opportunistic infections are extremely common in these patients.

The typical malignant circulating cells have moderately condensed nuclear chromatin, inconspicuous nucleoli, and a markedly irregular nuclear contour in which the nucleus is divided into several lobes.[258] These cells typically express the phenotype of helper/inducer T lymphocytes,[259-261] and the Tac antigen (CD25) that identifies the IL-2 receptor.[262] Variability in the expression of T3, T11, and T12 have been reported.[261] Clonal rearrangements of the Tβ receptor are identified in cells from patients with adult T cell leukemia/lymphoma.[173,175,226,239] The leukemic cells are reported to suppress B cell Ig secretion[263] by a complex mechanism involving induction of suppressor cells following activation of normal suppressor cell precursors.[261]

CLL and prolymphocytic leukemia. CLL is a monoclonal proliferation of SmIg-positive B lymphocytes.[264,265] Clonality of CLL has been demonstrated by expression of a single Ig light chain, either κ or λ, on the cell surface membrane.[266,267] More sophisticated techniques have confirmed clonality by showing unique immunoglobulin idiotype specificities,[268] a single pattern of glucose-6-phosphate dehydrogenase activity,[269,270] clonal chromosome abnormalities,[271] or immunoglobulin gene rearrangement.[157] The malignant B cell involved in CLL is an intermediately differentiated cell. The cell appears frozen in differentiation and does not mature to the final stage of B cell development, the mature plasma cell. However, recent data have demonstrated that in vitro treatment of these cells with phorbol esters or pokeweed mitogen can induce differentiation into mature immunoglobulin-secreting plasma cells.[272] In another study, this immunoglobulin secretion was preceded by a rapid increase in the level of mRNA coding for IgM, a predominantly secretory form of mRNA rather than a membrane form of mRNA.[273] This selection is similar to that seen in plasma cells, and the study clearly demonstrated at the molecular level that CLL cells consistently retain the capacity to differentiate to plasma cells and secrete immunoglobulin. Under certain circumstances, CLL cells stimulated in vitro with phorbol esters differentiate into cells with cytoplasmic protrusions and other characteristics of hairy cell leukemia.[274]

The B lymphocyte characteristic of CLL displays a relatively small amount of SmIg, estimated to be ~9,000 molecules per cell.[275] Relatively weak fluorescence of SmIg has been used to distinguish CLL from the leukemic phase of nodular and diffuse lymphocytic lymphomas and from prolymphocytic leukemia in which the cells generally display considerably more SmIg.[276,277] Immunoglobulin isotype analyses indicate that most CLL display a single heavy chain class; typically, μ or μ and δ. Less commonly, γ, α, or no heavy chain determinant is found. CLL cells display either κ or λ light chains but never both. Some data suggest that heavy chain switching can occur in B-CLL, which may indicate increasing maturity of the malignant cell.[277] Other studies indicate that CLL cells contain only μ or μ and δ and that γ is extrinsic and not synthesized by the leukemic cells.[278] Although there has been controversy as to whether CLL B cells contain CIg, the presence of cytoplasmic heavy chains (μ and δ) has been reported in most patients with CLL; no γ or α chains were detected.[279] B-CLL cells display receptors for mouse erythrocytes, a feature characteristic of immature B lymphocytes.[280] The cells also have the receptor for the Fc portion of IgG and complement with a relative increase of C'3d receptors (CR2) over C'3b receptors (CR1); this is typical of immature B cells.[281] B-CLL cells display several antigens, including Ia and human B cell antigens such as BA1, B1, B2, and B4. One unanticipated finding was that B-CLL cells display a 65-kd glycoprotein antigen previously thought to be restricted to T lymphocytes. This antigen was first recognized by using heteroantisera[282]; later, it was recognized with the T101 and equivalent monoclonal antibodies.[31-33] The precise meaning of this anomalous expression of a T cell antigen is unclear, although a normal B cell counterpart has been reported in human tonsil lymph nodes,[283] and stimulation in vitro of normal B cells with phorbol ester may induce expression of this antigen.[284] Recently, the TQ1 antigen, reported to define the inducer of suppression within the T helper subset, was identified on 60 of 75 B-CLL patients' cells.[285]

Rearrangement of immunoglobulin heavy and light chains has been reported as expected in B-CLL cells; however, rearrangement of the Tβ receptor has also been reported in ~10% of cases of B-CLL.[175] This is analogous to the reported Tβ rearrangement in 25% of non-T (pre-B) ALL, and again emphasizes that immunoglobulin and Tβ receptor rearrangement alone are not adequate to assign lineage.

In 3% to 10% of patients with CLL, the disease may evolve into a diffuse histiocytic lymphoma (Richter's syndrome). This is associated with loss of the TQ1 antigen.[285] Most data suggest that this evolution involves transformed follicular center B cells rather than histiocytes or macrophages. Some transformations represent evolution of the malignant clone with expression of the same monoclonal immunoglobulin and karyotypic abnormality present in the original CLL clone.[286] In other cases, the lymphoma cells have different markers and immunoglobulin gene rearrangements than those of the original CLL cells; these cases probably represent the concomitant development of a B cell lymphoma or a histiocytic malignancy in patients with CLL.[287,288]

Prolymphocytic leukemia (PL) is related to CLL and is also likely to be derived from cells from the medullary cords of the lymph node. Immunoglobulin gene rearrangements of heavy and light chains have been reported.[289] Patients with PL generally have extremely high blast counts and splenomegaly but lack significant lymphadenopathy. Prolymphoblasts likely are activated cells and appear morphologically immature, with a fine lacy nuclear chromatin and one to two nucleoli; they may contain intracytoplasmic granules. These cells generally have higher density SmIg than do CLL cells; they have Ia and B4 antigens and may form rosettes with mouse erythrocytes.[220] PL cells from 14 consecutive patients reacted with the FMC7 monoclonal antibody that recognizes an antigen found on one half of normal B lymphocytes, whereas cells from only 5 of 20 patients with CLL reacted with this antibody.[7]

Approximately 5% of cases of CLL and PL result in a malignant proliferation of T rather than B cells. These cells react with T antisera and anti-T monoclonal antibodies reflecting the phenotypes of mature T lymphocytes; they lack SmIg and other B cell markers.[290,291] Many of these patients have diffuse organ and skin involvement.[290] T-CLL cells have been reported to have either helper or suppressor surface markers.[202,292,293] One patient's cells rosetted with sheep erythrocytes expressed the Leu-2 (suppressor-associated) antigen and also had SmIg and CIg (IgMλ).[294] Other instances in which the leukemia/lymphoma cells expressed characteristic features of both B and T lymphocytes have also been reported.[295]

Hairy cell leukemia. Hairy cell leukemia (leukemic reticuloendotheliosis) is characterized by invasion of the bone marrow and spleen by morphologically distinct mononuclear cells with "hairy" cytoplasmic projections.[296] These cells usually contain an isoenzyme of acid phosphatase (isoenzyme 5) that is resistant to tartrate; this isoenzyme is not unique to hairy cells. Surface markers of hairy cells are most consistent with a monoclonal proliferation of B lymphocytes.[297-300] SmIg with a single light chain is frequently identified,[299,300] as are B cell-associated antigens including Ia, B1, FMC-1, FMC-7, and sometimes BA-1.[299-301] The PCA-1 antigen (but not the PC-1 antigen) typically on plasma cells is identified on hairy cells; these data suggest that hairy cells may be pre-plasma cells.[302] Perhaps the most convincing evidence for the B cell origin of hairy cells comes from studies of immunoglobulin genes which indicate clonal rearrangement of heavy chain genes and at least one light chain gene.[303,304] Most cases of hairy cell leukemia demonstrate the 53-kd to 57-kd Tac antigen (IL-2 receptor) typically identified on select T cell malignancies and activated T cells.[304] Another antigen with a mol wt of 52 to 67 kd, designated HC-2, appears to be restricted to hairy cell leukemia cells.[305,306] The αS-HCL 1 antibody reacts with normal B lymphocytes and B cell malignancies, including hairy cell leukemia.[109] The αS-HCL 3 reacts with normal monocytes and AML cells but is restricted to hairy cell leukemia among the lymphoid malignancies.[109] Approximately 2% to 3% of normal peripheral blood B lymphocytes express this antigen; these appear to be activated B cells.

Myeloma and related disorders. The malignant B cells of Waldenström's macroglobulinemia, heavy chain disease, and multiple myeloma represent a further step in the maturation of medullary cord B cells.[264] Like CLL cells, cells from patients with Waldenström's macroglobulinemia express SmIg and Ia, B1, and B4 antigens.[11] Unlike CLL cells, however, these cells express the PCA-1 antigen but do not express the B2 antigen nor do they rosette with mouse erythrocytes.[4,220] The plasma cell and its malignant counterpart, the myeloma cell, represent the most differentiated B lymphocytes. These cells synthesize large quantities of immunoglobulin and have CIg, but usually lack SmIg and the Ia, B1, B2, and B4 antigens.[307] Plasma cells and myeloma cells, like other mature B lymphocytes, usually lack CALLA, but a recent study has suggested that rare cases of CALLA-positive myeloma represent an aggressive subtype with a poor prognosis.[308] Plasma cells and myeloma cells stain intensely with the OKT10 monoclonal antibody as well as the anti-PCA-1 and anti-PC-1 antibodies.[309,310]

CORRELATES OF CELLULAR DIFFERENTIATION WITH LYMPHOID MALIGNANCIES

Substantial data suggest that the phenotypes of most leukemia cells are not unique but reflect characteristics of normal cells. None of the surface markers we have reviewed are leukemia specific; all can be identified on normal as well as malignant cells. Most of the monocyte, granulocyte, and lymphocyte antigens are found on mature and immature cells. However, CALLA, BA-2, and RFB-1 are expressed primarily on immature bone marrow cells. This observation is consistent with the phenotypes of leukemic cells, since the CALLA and BA-2 antigens are present on primitive leukemia cells (ALL and lymphoid blast crisis of chronic myelogenous leukemia cells) but only rarely on more mature leukemia or lymphoma cells. Distribution of the reactivity of the monoclonal T antibodies is likewise consistent with this hypothesis. The most primitive thymocyte markers, OKT9 and OKT10, are found on most T-ALL cells, whereas T3/Leu-4, T8/Leu-2, and T4/Leu-3, which are found on mature thymocytes and circulating T lymphocytes, are more often identified on more mature T cell leukemias.

A proposed scheme of normal lymphoid differentiation is presented in Fig 2. This scheme is based on the concept that the phenotype of normal lymphoid cells at each level of differentiation can be detected from the phenotype of its malignant counterpart. Although some malignant cells may have an aberrant phenotype, the data presented suggest that

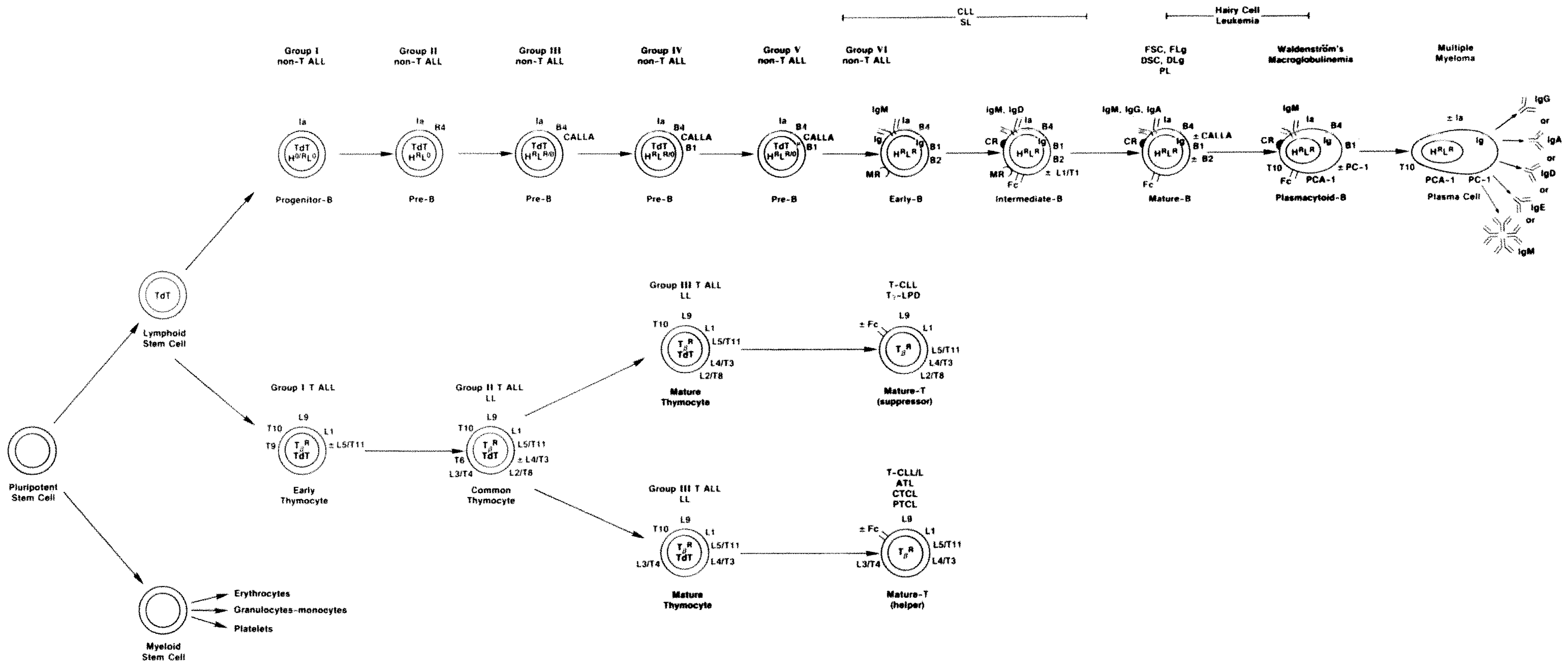

Fig 2. Schematic representation of human lymphoid differentiation and related lymphoid malignancies. ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; SL, malignant lymphoma, small lymphocytic; FSC, malignant lymphoma, follicular small cleaved cell; FLg, malignant lymphoma, follicular large cell; DSC, malignant lymphoma, diffuse small cleaved cell; DLg, malignant lymphoma, diffuse large cell; PL, prolymphocytic leukemia; LL, lymphoblastic lymphoma; Tγ-LPD, Tγ-lymphoproliferative disease; ATL, adult T cell leukemia/lymphoma; CTCL, cutaneous T cell lymphoma; PTCL, peripheral T cell lymphoma; TdT, terminal deoxynucleotidyl transferase; H, heavy chain; L, light chain; O, germline configuration; R, rearranged gene; T_β^R, clonal rearrangement of the Tβ receptor; MR, mouse rosette; CR, complement receptor.

most malignant lymphoid cells reflect the phenotype of a normal lymphocyte. The proposed phenotype of the progenitor B lymphocyte probably has the same surface markers as the group I non-T-ALL and represents the earliest identifiable B cell. This cell expresses the Ia antigen, but no other B cell-associated antigens. The next level of B cell differentiation coincides with the group II non-T-ALL; heavy but not light chain immunoglobulin genes are rearranged. At the next level of B cell differentiation, the cells express CALLA and light chain gene rearrangements occur; this coincides with group III non-T-ALL. With sequential steps in B cell differentiation, the B1 antigen is expressed, followed by Cμ and then SmIg. At the next level of B cell differentiation, the B cell acquires the B2 antigen and the receptor for mouse erythrocytes; both SmIg and CIg are present. Most CLL cells—and malignant lymphoma, small lymphocytic type cells—express the phenotype of intermediate B lymphocytes. The cells express receptors for complement and the Fc portion of IgG, Leu-1, in addition to the surface markers identified on more primitive B cells. At this level of differentiation, there is low-density SmIg. The maturing B cells express high-density SmIg (IgM, IgG, or IgA) without B2 or mouse erythrocyte receptors. The malignant counterparts of the mature B cell are the follicular small cleaved and large cell lymphomas and the diffuse small cleaved and large cell lymphomas and PL cells. At the next step of maturation, the plasmacytoid B cell secretes Ig, usually of the IgM subclass, and expresses new surface membrane antigens including OKT10, PCA-1, and PC-1. It has recently been demonstrated that hairy cell leukemia falls somewhere between the mature B cell and plasma B cell; they also express the PCA-1 antigen.[302] The plasma cell, the most differentiated B lymphocyte, expresses the same phenotype as myeloma cells. Although these cells have CIg, produce immunoglobulin, and express OKT10, PC-1, and PCA-1, they lose other surface membrane markers, including SmIg, Ia, and B cell antigens.

T cell differentiation follows a distinct pathway. Early thymocytes (stage I) express Leu-9, Leu-1, T9, T10, and often T11/Leu-5 (sheep erythrocyte receptor); this phenotype probably represents the malignant counterpart of group I T-ALL cells. A case has been made that the stage I thymocyte has not yet rearranged the Tβ receptor gene[212]; this remains to be confirmed. The common thymocyte (stage II) no longer expresses T9; it gains T6 antigen and simultaneously expresses the helper-associated (T4/Leu-3) and suppressor-associated antigens (T8/Leu-2). This cell clearly rearranges the Tβ gene, confirming that Tβ rearrangement precedes surface membrane expression of the T3–Ti complex.[212] This cell corresponds to the phenotype of group II T-ALL and some lymphoblastic lymphomas. Subsequently, the cells lose either the helper-associated or suppressor-associated antigens. This is equivalent to group III T-ALL or some cases of lymphoblastic lymphoma. In the final stage of maturation, the suppressor-associated cell (T8/Leu-2) may express the receptor for the Fc portion of IgG as well as the surface markers previously attributed to the mature thymocyte (including the T3–Ti complex), coinciding with the phenotype of some T-CLL cells and chronic Tγ lymphoproliferative disease. The helper-associated mature T lymphocyte (T4/Leu-3), on the other hand, may express the receptor for the Fc portion of IgM and coincides with the phenotypes of some T-CLL, adult T cell leukemia/lymphoma, cutaneous T cell lymphoma, and peripheral T cell lymphoma.

CLASSIFICATION OF THE NONLYMPHOID LEUKEMIAS AND LYMPHOMAS

Hodgkin's disease. Hodgkin's disease (HD) is a malignant neoplasm of uncertain cellular origin characterized by the appearance of distinctive binucleate or multinucleate giant cells (Reed-Sternberg cells, RSCs) and their mononuclear variants (Hodgkin's cells, HCs).[311] The malignant nature of this disease is suggested by cytogenetic studies that have shown a clonal distribution of chromosomal aneuploidy.[312–314] Considerable debate has arisen as to what constitutes the malignant cell of HD. However, most investigators now agree that the RSCs or HCs (a subset constituting a minute fraction of the tumor mass) represent the neoplastic cell population.[315] The normal cellular counterpart from which RSCs and HCs arise has not yet been identified.[315]

Investigators have used morphology (light and electron microscopy), cell culture, and immunohistochemistry in an attempt to characterize the nature of the RSCs and HCs. Based on these observations, it has been argued that HD arises from the T lymphoid,[316,317] B lymphoid,[318–328] or myeloid-macrophage lineages.[311,329–331] Although this controversy is unresolved, the application of immunologic marker analysis has contributed to our further understanding of the disease, and several general statements can be made. First, with few exceptions, most observers have failed to detect the uniform expression of T cell surface markers (as defined by polyclonal and monoclonal reagents)[328,332–337] by RSCs or HCs, suggesting that these cells are not of T lymphocyte origin. RSCs and HCs have been shown to express the Tac antigen (IL-2 receptor)[338]; in two reports, these cells were found to be T9 positive (transferrin receptor).[332,334] Neither receptor-associated marker is restricted to the T cell lineage.[304,339] Second, although the detection of SmIg or CIg in RSCs and HCs favors a B cell origin,[318,319,322] the expression of these determinants is often polyclonal,[321,340–344] which suggests that immunoglobulin is adsorbed onto RSC and HC cells rather than being synthesized by the malignant cell.[340] There are no convincing data that RSCs or HCs produce immunoglobulin. Immunologic staining of RSCs and HCs for the expression of B cell differentiation antigens has produced conflicting results.[332,333,345] In an interesting case of B cell HD,[346] a patient with nodular sclerosing HD developed a terminal leukemic phase. The circulating HCs expressed the B1 and B4 antigens and had cytoplasmic μ heavy chains and a clonal rearrangement of heavy and light chains (consistent with a B cell origin). Substantial data, however, favor a myeloid-macrophage origin for HD.[340,341,344,347–349] This conclusion is based on the demonstration of nonspecific esterase (NSE) and acid phosphatase, α-1-antitrypsin and α-1-antichymotrypsin, muramidase, lectin-binding properties, and the variable expression of Fc and C3 receptors on

RSC and HC cells. Although short-term cell lines believed to be derived from RSCs demonstrate weak phagocytic activity, and one line was reported to synthesize IL-1,[347,349] other established cell lines have not uniformly shown these activities.[350] In most instances, RSCs and HCs do not react with antibodies to monocytes,[318,332–336,345,350] although in one report a substantial number of biopsy specimens contained RSC and HC positive for markers characteristic of late granulocytic maturation (TU5, TU6, TU9).[333,351] Based on Ia expression[318,332,336,345] and characteristic cytochemical features, other authors have suggested that the cell of origin for HD is a "reticulum cell" (either a dendritic cell or an interdigitating reticulum cell).[336,352] Finally, some data suggest that the RSCs and HCs represent a subset of activated lymphoid cells of either T or B lymphoid origin. This conclusion is based on an immunologic analysis in which RSC and HC uniformly expressed the Ki-1 marker (35 of 35 biopsy specimens of all histological subtypes) as defined by a monoclonal antibody raised by immunization against an established HD cell line.[353] Among normal cells, Ki-1 is expressed by T and B lymphocytes activated in vitro by various stimuli that also induce interleukin-2 (IL-2) receptor expression.[337] In situ staining of biopsy specimens from nonneoplastic and reactive tissues demonstrated Ki-1 expression by a population of normal perifollicular lymphoid cells (lymph node and spleen) and variable degrees of expression by abnormal lymphoid cells in cases of angioimmunoblastic lymphadenopathy and lymphatoid papulosis. Among 290 cases of non-Hodgkin's lymphoma, Ki-1 expression was observed in 19 cases of peripheral T cell lymphoma and in 45 cases of diffuse large cell lymphoma (including 35 specimens expressing T cell surface markers and 7 bearing B cell antigens). These results suggest that Ki-1 is a lymphoid activation antigen that identifies a group of large lymphoid cells in normal and neoplastic tissues (including RSCs) that remains poorly characterized. Another monoclonal reagent, HeFi-1, is similar if not identical to Ki-1.[354]

In summary, the cellular origin for HD remains unclear. Although Ia and T9 antigen staining of RSC and HC cells have been reported, Ki-1 antigen expression may prove to be the most useful immunologic marker for this disease.

RSCs and HCs constitute only a small portion of cells within the tissue of Hodgkin's disease. Recent efforts are directed toward characterizing the remaining cells and have been recently reviewed.[355] Use of in situ techniques has demonstrated that lymphoid tissues involved with HD appear to be heterogeneous in immunohistologic make-up; some cases demonstrate numerous T lymphocytes with few B cells, whereas others exhibit prominent follicles of polyclonal B lymphocytes and only small numbers of T cells within these follicles. In two studies,[335,355] these B cell-rich cases were of the lymphocyte-predominant type. In specimens containing T lymphocytes, RSCs and HCs tend to appear in areas of heaviest T cell infiltration, suggesting a relationship. Many cells within areas of T cell infiltration are Ia or T10 positive, suggesting that these T lymphocytes are activated. Several investigators have demonstrated that most HD-associated T lymphocytes are of the helper cell subset.[332,335,355–357] Genotyping for immunoglobulin and T cell receptor rearrangements may lead to a better understanding of the cellular origin of this disease.

Malignant disorders of macrophages. Several malignant diseases of macrophages (or histiocytes) have been described.[358,359] The term histiocytic lymphoma, used in the Rappaport classification,[215] encompasses a heterogeneous group of neoplasms of large transformed lymphocytes and, rarely, of macrophages.[360,361]

The malignancies of macrophages (histiocytosis X) are heterogeneous.[362] Clinical presentations include solitary benign eosinophilic granuloma, Hand-Schuller-Christian disease, and histiocytic medullary reticulosis; the latter is a generalized systemic disorder characterized by fever, wasting, hepatosplenomegaly, variable lymphadenopathy, and progressive pancytopena due to diffuse tissue invasion by malignant macrophages.[363] The equivalent disease in children is sometimes referred to as Letterer-Siwe disease. Because of morphologic and ultrastructural similarities between malignant macrophages and epidermal Langerhans cells, it has been proposed that these diseases represent a proliferative disorder of Langerhans cells.[364–366] Both cell types possess receptors for C3 and the Fc portion of IgG[367] and express the T6 and Ia antigens.[368,369] The malignant macrophages also express the OKM1 and other macrophage surface markers.[370] An unexpected and unexplained finding was the presence of the T4 antigen in this disorder.[368]

AML. AML is a clonal malignancy of myeloid progenitor cells resulting in excessive proliferation and accumulation of immature hematopoietic elements. The subtypes of this disease are generally classified according to the morphologic similarity of the leukemic cell population to normal myeloid precursors, eg, acute myeloblastic leukemia, acute promyelocytic leukemia, acute monoblastic leukemia, and acute erythrocytic leukemia.

Monoclonal antibodies have been evaluated for reactivity against AML cells (Tables 9 and 10). In all cases, these antibodies identify determinants expressed by either normal circulating myeloid cells or bone marrow progenitors. None of these reagents recognizes a leukemia-specific determinant and, with few possible exceptions,[371–373] attempts to generate leukemia-specific antisera have been unsuccessful. Studies of the reactivity of antimyeloid cell antibodies for AML cells have raised several issues: (a) whether these reagents are specific in their reactivity for myeloid *v* lymphoid leukemia cells; (b) whether antibody reactivity correlates with classification using the FAB nomenclature; (c) whether surface marker expression by myeloid leukemia cells corresponds to stages of normal myeloid differentiation and, if so, whether this is of prognostic significance; (d) whether myeloid leukemia cells in a patient are homogeneous in their expression of surface markers; (e) whether leukemia progenitor cells defined by their ability to form leukemic colonies in vitro exhibit the same antigenic phenotype as their progeny in bone marrow and blood; and (f) whether monoclonal antibodies that identify antigens expressed by myeloid leukemia cells can be used for immunotherapy.

AML v the lymphoid leukemias. Because of differences in prognosis and therapy, it is important to distinguish between AML and the lymphoid leukemias. Although differ-

Table 9. Use of Myeloid Surface Markers to Discriminate Between Acute Myeloid and Lymphoid Leukemia

	Myeloid Leukemia			Lymphoid Leukemia			
Monoclonal Antibody	AML	CML (MBC)	Total Myeloid	ALL	CML (LBC)	Total Lymphoid	References
Mo1/OKM1	63 (228)*	57 (23)	62 (251)	0 (82)	0 (11)	0 (92)	66,106,118,124,389,419
MY7	76 (97)	92 (13)	78 (110)	1 (109)	0 (13)	1 (122)	118,119,419
MY8	53 (73)	7 (15)	45 (88)	0 (82)	0 (11)	0 (83)	118,119,419
MY9	85 (97)	92 (13)	85 (110)	2 (109)	0 (13)	2 (122)	70
VIM-2	91 (66)	93 (30)	92 (96)	5 (60)	0 (11)	4 (71)	105
VIM-D5	68 (116)	88 (8)	69 (174)	2 (88)	NR	2(88)	389,418

MBC, myeloid blast crisis; LBC, lymphoid blast crisis.

*Percentage of patients positive (total number of patients tested). A patient is considered positive for a given marker if >20% of malignant cells bind the monoclonal antibody.

ences in morphology and histochemistry often lead to the correct diagnosis, the distinction between immature variants of AML and ALL is not always evident. Monoclonal reagents that identify antigens expressed by myeloid but not lymphoid leukemias (or vice versa) would therefore be important. Six monoclonal antibodies have been extensively tested for reactivity to myeloid and lymphoid leukemia (Table 9). Each of the six antigenic determinants defined by these antibodies (Mo1/OKM1, MY7, MY8, MY9, VIM-2, and VIM-D5) is expressed by more than one half of patients with AML (53% to 91%), defined as antibody binding by >10% to 20% of malignant cells in each patient. The myeloblasts of patients with myeloid blast crisis of CML demonstrate similar frequencies of expression for these determinants except for MY8. Conversely, expression of these antigens on acute lymphoid leukemia cells (including the T and B cell variants of ALL and chronic myelogenous leukemia (CML) lymphoid blast crisis) is rare. Clearly, these monoclonal reagents can complement other tests in the differential diagnosis of AML versus ALL. The accuracy of immunologic diagnosis can be extended by using more than one antimyeloid reagent in conjunction with antibodies that detect antigenic determinants uniquely expressed by B or T lymphoid leukemias (anti-CALLA, B1, B4, OKT3, Leu-4, etc.) (see above). Occasionally, however, this approach has produced seemingly disparate results, with the detection of leukemia cells with myeloid and lymphoid differentiation markers.[374–376] These rare situations may reflect the existence of biopotential clones of malignant cells expressing features of more than one lineage.[377]

Correlation between surface marker phenotype and FAB classification. There are several types of AML differ in morphology, histochemistry, and surface marker expression. Classifications have been proposed to identify these types, based on the hypothesis that this information may be of prognostic and therapeutic significance. The FAB group classification, which relates the morphologic appearance of leukemic cells to presumed normal hematopoietic counterparts, is widely used.[185,186,378,379] Seven subtypes of AML (M1 through M7) are identified: M1 and M2 represent undiffer-

Table 10. Correlation Between Myeloid Surface Marker Expression and FAB Classification System

		FAB Classification					
Monoclonal Antibody		M1	M2	M3	M4	M5	References
VIM-2	(M + N)*	73 (15)†	96 (23)	83 (6)	100 (16)	100 (6)	105
R1B19	(N)	40 (10)	44 (9)	13 (8)	48 (27)	38 (8)	67,68
S4-7	(M + N)	50 (10)	56 (9)	25 (8)	70 (27)	78 (9)	67,68
PM 81	(M + N)	90 (10)	50 (2)		86 (7)	100 (3)	97,122
MY9	(M)	85 (54)‡		100 (6)	81 (31)	83 (6)	70
MY7	(M + N)	78 (54)		67 (6)	81 (31)	50 (6)	118,119
Mo5	(M + N)	50 (38)			74 (27)‡		95
MOP9	(M)	17 (6)	13 (8)	0 (3)	100 (7)	100 (10)	389
AML-2-23	(M + N)	0 (12)	0 (2)		78 (9)	100 (5)	121,122
MY4	(M)	25 (36)		0 (3)	52 (25)	100 (6)	119
UCHM1	(M)	6 (17)			92 (24)	100 (16)	66
MY8	(M + N)	36 (39)		33 (3)	76 (25)	83 (6)	118,119
Mo1/OKM1	(M + N)	39 (124)		100 (3)	91 (101)		66,106,118,124,389
Mo2	(M)	14 (65)			45 (31)		124
VIM-D5	(N)	29 (38)	71 (52)	67 (12)	88 (40)	92 (24)	389,418
82H5	(N)	0 (5)	69 (13)	100 (4)	100 (6)	100 (7)	83

*Antigen expression by peripheral blood monocytes (M) or neutrophils (N).

†Percentage of patients positive (total number of patients tested).

‡Patients with M1 and M2 or M4 and M5 leukemia combined.

entiated and differentiated myeloblastic leukemia; M3, promyelocytic leukemia; M4 and M5, myelomonocytic and monocytic variants; M6, erythroleukemia; and M7, megakaryocytic leukemia. Although some investigators have reported briefer remissions or lower response and survival rates in patients with the M5 variant,[380-382] fewer remissions in erythroleukemia (M6),[380] or longer remissions in promyelocytic leukemia (M3),[380-382] these observations are controversial.[186,381-383] With the development of immunologic reagents that detect antigenic markers expressed by normal and leukemic myeloid cells, analyses have been undertaken to compare the FAB system with patterns of surface marker expression. Table 10 indicates 16 monoclonal reagents whose relative reactivity against FAB-classified AML variants can be critically assessed. Within the first group of seven antibodies (VIM-2, R1B19, S4-7, PM81, MY9, MY7, and Mo5), no clear distinction exists in antigen expression by cells in each of the five FAB variants (M1-M5; too few patients with the M6 and M7 variants were examined to draw conclusions). The frequency of expression by patients in each subclass is generally 50%. In the second group of seven antibodies (MOP9, AML-2-23, MY4, UCHM1, MY8, Mo1/OKM1, and Mo2), there is a trend toward higher frequency of antigen expression among individuals whose leukemia cells display monocytic differentiation (M4 + M5). In the case of VIM-D5 and 82H5, only undifferentiated M1 cells have a lower frequency of expression. None of these reagents demonstrates preferential binding frequency to M1 and/or M2. One antibody, VIE-64, which binds to glycophorin A, displays relative binding specificity toward M6 variant cells.[384] Monoclonal antibody SFL 23.6 has a well-defined reactivity restricted to the erythroid lineage including erythroleukemia cell lines and should be useful in distinguishing M6.[125] Monoclonal antibodies against platelet glycoproteins Ib, IIb/IIIa, and aIIIaa, for factor VIII-related antigen can be used to identify megakaryoblasts.[378] With these possible exceptions, the degree of correlation between surface marker expression and the criteria for FAB classification is not convincing.[385,386]

Subclassification of AML according to differentiation-associated phenotypes as identified by monoclonal antibodies. Because there is controversy over whether the FAB classification system provides prognostic information, alternative classifications have been proposed. In a surface marker analysis of 70 patients with AML, Griffin and co-workers[119] identified four phenotypes based on patterns of surface antigen expression that correlated with phenotypes displayed by myeloid cells during normal differentiation. Group I AML cells (21% of patients) expressed the antigenic phenotype of the CFU-C–committed myeloid progenitor cell (Ia and MY7-positive); group II cells (26%) displayed the phenotypic characteristic of normal myeloblasts (MY7, Ia, and Mo1/OKM1 and My8-positive); group III cells (8%) had a phenotype featured by normal promyelocytes (MY7 and Mo1/MY8-positive; Ia-negative); and group IV cells (45%) with the phenotype of promonocytes and monocytes (MY4, MY7, MY8/Mo1, and Ia-positive). Within these four differentiation-related groups there was considerable morphologic heterogeneity: although all three of the promyelocytic leukemia (M3) patients were in group III and all six monocytic leukemia (M5) patients were in group IV, the myeloid leukemia (M1 and M2) and myelomonocytic leukemia (M4) patients were dispersed throughout all four groups, with a tendency for myeloid patients to be in groups I, II, and III, and myelomonocytic patients to be in groups II and IV. The preliminary finding of a larger prospective analysis involving over 200 patients demonstrates significant differences among these phenotypic groups with respect to complete response rate and disease-free survival (J.D. Griffin, personal communication). Moreover, expression of certain markers appears to be of independent prognostic significance: AML patients with MY7-positive leukemia exhibit a worse prognosis than do MY7-negative patients; the expression of monocyte antigen MY4 is also predictive of a poor response. Several studies of the biological implications of surface marker phenotype in AML are in progress.

A scheme for myeloid differentiation is shown in Fig 1. In an attempt to account for the FAB M4 leukemic cell (bearing features of both granulocytic and monocytic differentiation), Ball and Fanger[122] have proposed that the normal M4 counterpart is an intermediate bipotential precursor cell capable of differentiating along either the monocytic or granulocytic path of differentiation. They further suggest that the myeloblast (M1 and M2), the progenitor of the M4 cell, is likewise bipotential. Given the ability of the promyelocytic leukemia cell line HL-60 to undergo subsequent differentiation toward mature monocytes or neutrophils depending on the nature of the inducing stimulus,[387,388] it appears that the normal promyelocyte is not irreversibly committed to granulocytic maturation. Although these hypotheses are consistent with some experimental observations, considerable additional data are required.

Heterogeneity of surface marker expression by malignant AML cells. Most studies of AML indicate considerable heterogeneity in leukemic cell surface marker expression between patients as well as within a given individual.[62,67,69,95,114,118,122,386,389] Typically, a patient is classified as positive for the expression of a marker if >10% to 20% of the patient's leukemia cells display the determinant. Although certain antigens tend to be expressed by >50% of the leukemic cells of given individuals, variability is considerable. If surface marker expression correlates with the level of myeloid differentiation, these data suggest that the leukemic population is heterogeneous.

AML likely arises from leukemic myeloid progenitor cells, which in some cases can be grown in vitro in semisolid medium.[390-392] The clonogenic leukemia cells (L-CFC) are, by definition, capable of limited proliferation (with a subset capable of self-renewal), a feature that distinguishes them from most leukemia cells that are terminally differentiated.[392,393] Several groups have recently investigated the surface marker characteristics of L-CFC and compared them with the total leukemia population.[70,114,394,395] The surface-marker phenotype of the total leukemia population, as determined by immunofluorescence analysis, may not predict the phenotype of the L-CFC as measured by inhibition of L-CFC growth after antibody-dependent, complement-mediated lysis. In general, the L-CFC has a pattern of antigenic expression that is more "immature" than that of the predominant phenotype of the total population. L-CFC have been subclassified using multiple markers whose expression on normal CFU-GEMM (Ia, MY9, S3-13, S8-6), early (day 14) CFU-GM (Ia, MY9, PM-81, S3-13, S8-6, S4-7), and late (day 7) CFU-GM (all of the preceding markers plus AML-2-23 and R1B19) are known. Three phenotypically distinguishable levels of differentiation have been identified.[114,394] The degree of maturity, as based on morphology (FAB classification) and expression of "later stage" antigens (beyond the CFU-GM: Mo1, MY3, Mo2) of the total leukemia population, tends to correlate with the L-CFC maturation level (eg, CFU-GEMM level L-CFC are associated with M1 morphology and lack expression of late antigens), suggesting a limited potential for terminal differentiation[114,395] These data suggest that L-CFC are a distinct subset of clonogenic cells among the total leukemia population; these cells may arise at multiple points along the pathway of early myeloid differentiation.

CML. CML is a myeloproliferative disorder characterized by a consistent chromosomal abnormality, the Philadelphia (Ph^1) chromosome. The Ph^1 chromosome results from a reciprocal translocation between chromosomes 9 and 22 designated t(9;22).[396] This translocation results in the transfer of the c-*abl* oncogene from chromosome 9 to the Ph^1 chromosome and the variable reciprocal translocation of c-*sis* from chromosome 22 to 9.[397] The target for leukemic transformation (Fig 3) appears to be at the level of the pluripotential stem cell, since the Ph^1 chromosome is present in all hematopoietic elements of patients with CML, including B and T cells. [398-404] The clonal origin of CML is further indicated by analysis of patterns of expression of glucose-6-phosphate dehydrogenase (G6PD), and adenylate kinase isoenzyme.[398,402] In the chronic phase of the disease, CML is characterized by an overproduction of relatively mature granulocytes. After a variable period of time, with a median of 3 years, most patients enter an acute phase (blast crisis) in which maturation no longer occurs. The acute phase resembles acute leukemia. Approximately one-third of patients with acute-phase CML demonstrate cells with lymphoid features that include the expression of TdT, CALLA, Ia, B1, rarely $C\mu$ and rearrangements in immunoglobulin heavy and light chain genes.[403,405-411] Rare cases of lymphoid acute phase with T cell markers have also been reported.[412-414] Acute phase CML involving myeloid cells is heterogeneous; typically the cells resemble myeloblasts, but erythroblasts, megakaryoblasts, and monoblasts can also be observed. Distinction between lymphoid and myeloid acute phase is important because patients with lymphoid blast crisis may respond to chemotherapy with vincristine (V) and prednisone (P).[410,415-417] In making this diagnostic distinction, the characteristic expression of several myeloid markers (MY7, MY9, VIM-2, and Mo1/OKM1) on myeloid blast crisis cells and their lack of expression by lymphoid blast crisis cells (Table 9) provide information complementary to assays for the detection of CALLA, B1, TdT, and CIg.[38,66,70,105,106,118,119,124,409,418,419]

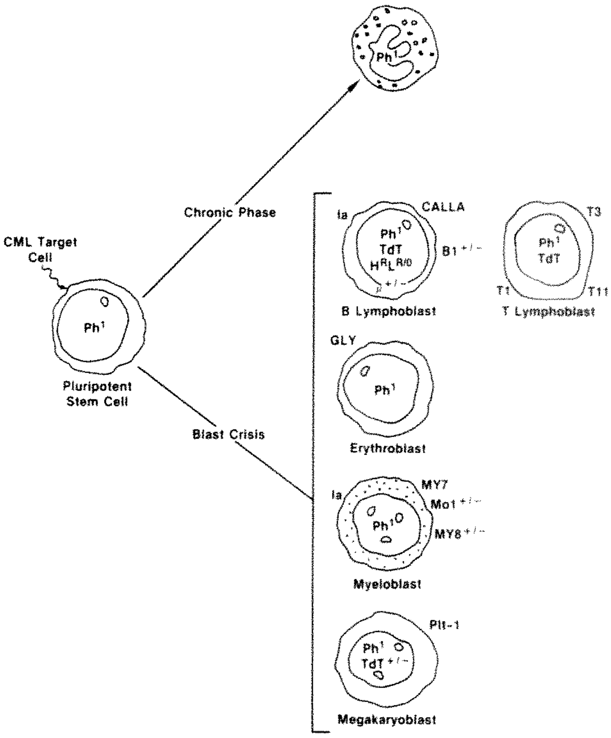

Fig 3. Schematic representation of the origin of chronic and blast crisis phase of chronic myelogenous leukemia (CML) from the target pluripotent stem cell. Phenotypes for various forms of CML blast crisis (based on data of Griffin et al[419]) are indicated. Ph^1, Philadelphia chromosome; TdT, terminal deoxynucleotidyl transferase; GLY, glycophorin A.

Surface marker analysis may allow further discrimination among the heterogeneous presentation of CML acute phase. Four phenotypes were identified in 30 patients with this disorder based on antigen expression of normal myeloid, erythroid, megakaryocytic, and lymphoid cells.[419] The cells of ten patients exhibited a phenotype corresponding to an immature myeloid cell (Ia, MY7, and Mo1-positive); all ten were negative for CALLA, B1, and TdT. These cases were felt to represent "myeloid" blast crisis; none of these patients responded to treatment with V and P. Cells from 11 patients expressed a phenotype similar to acute (early B) lymphoblastic leukemia cells (TdT, Ia, CALLA, and B1-positive; MY7 and Mo1-negative); six of nine evaluable patients had a complete response to V and P. Cells from one patient had the phenotype of erythroleukemia (glycophorin A-positive); another patient's cells expressed the phenotype of megakaryocytic leukemia (Plt-1-positive); one patient's cells had features of both myeloid and lymphoid blasts on different cells. Cells from six patients did not express surface markers characteristic of any lineage; these were termed "undifferentiated"; these cases were heterogeneous in expression of TdT; no complete responses to V and P were observed. Thus, in terms of response to V and P therapy, surface marker analysis provided useful prognostic information.

In another series of 45 patients with CML in blast crisis, 28 patients were classified as having "myeloid" blast crisis on the basis of reactivity with at least one of six antimyeloid monoclonal reagents (including VIM-D5 and VIM-2) and no surface expression of T or B cell immune markers or

TdT.[420] Among these myeloid cases, however, only 11 patients expressed granulomonocytic antigens exclusively; the blast cells of 17 patients were additionally positive for platelet/megakaryocyte markers (16 cases) and/or erythroid determinants (three cases). Whether the *same* blast cell co-expressed myeloid, megakaryocytic, or erythroid antigens was not determined. Fourteen patients were classified as having "lymphoid" blast crisis with the phenotypic pattern of CALLA-positive ALL (10 cases), pre-B cell ALL (three cases), or "Null" ALL (one case). Two patients demonstrated a mixed myeloid and lymphoid blast cell phenotype, and a single patient was unclassifiable. Sixteeen of these 45 patients were tested serially during the course of their illness and three demonstrated phenotypic changes. The immunological diagnosis of lymphoid blast crisis was associated with a higher rate of remission than was myeloid blast crisis (57% *v* 4%, respectively) and a longer median survival.

Certain patients with the Ph^1-chromosome are first diagnosed in the acute phase without a preceding history of a chronic phase.[415] Either a chronic phase never existed or it was never detected. Surface-marker analysis may be as useful in the subclassification of these patients' cells as it is in the more typical acute phase that is preceded by a chronic phase.

MONOCLONAL ANTIBODY THERAPY

Several investigators[421–431] have attempted to treat lymphoid or myeloid leukemias with monoclonal antibodies. In some studies, patients with advanced B cell-derived CLL received T101 monoclonal antibody.[421,422] T101 could be safely infused and led to transient reductions in circulating leukemia cells; there was, however, no sustained effect on the bone marrow, involved lymph nodes, or other organs. This therapy resulted in some intravascular cell injury, but destruction in the spleen, liver, and lungs was probably more important. Similar results have been reported in patients with adult T cell leukemia/lymphoma, ALL, and AML treated with other monoclonal antibodies.[423–425] Patients with cutaneous T cell lymphoma who received T101 or anti-Leu-1 have had only transient improvement in skin lesions and lymphadenopathy.[426–429] Side effects of monoclonal antibody therapy are usually minor. Respiratory distress following the rapid infusion of monoclonal antibody has been described,[422] and some patients have demonstrated transient elevation of serum creatinine and hepatic enzymes.[423]

Monoclonal antibody therapy has several shortcomings that must be addressed. First, treatment with antibodies such as T101 results in modulation of the antigen from the cell surface, which prevents antibody binding to the tumor cells. The T101 antigen-antibody complex is pinocytosed into the cytoplasm,[430] a phenomenon that might be advantageous when drugs or toxins are linked to the antibody to enhance its cytotoxicity. Antigen in the circulation poses another potential problem because it might prevent the antibody from reaching the tumor cells. Furthermore, murine antibodies can stimulate production of human anti-mouse antibodies which lead to antibody neutralization. This situation may be correctable by treatment with high initial doses of antibody ($>$ 500 mg) or by simultaneous treatment with immunosuppressive drugs to induce tolerance. In addition, the heterogeneity of antigen expression of tumor cells may necessitate therapy with more than one antibody. Clearly, monoclonal antibody therapy for leukemia and lymphoma is in its earliest stages.

An interesting therapeutic approach with monoclonal antibodies involves the use of anti-idiotype monoclonal antibody reactive with the idiotype of the immunoglobulin on malignant B cells. Such an antibody is by definition specific for a patient's tumor cells. A patient with B cell lymphoma in an accelerated phase who was unresponsive to conventional therapies was treated with an IgG_{2b} anti-idiotype monoclonal antibody.[431] Following eight intravenous (i.v.) infusions, the patient entered a complete remission that has been sustained for $>$3 years. Results were less impressive in other lymphoma patients treated with this approach with ~50% achieving short-lived partial remissions.[432,433] We developed several monoclonal anti-idiotype antibodies to cells from patients with leukemia and lymphoma.[268] The first patient to undergo treatment had advanced CLL. Sequential anti-idiotype monoclonal antibody therapy with IgG_{2b} and IgG_1 antibody provided no benefit. His therapy was limited because of circulating idiotype immunoglobulin that blocked the binding of the anti-idiotype antibody to the leukemia cells. We were able to reduce the circulating idiotype sufficiently with extensive plasmapheresis.

Although anti-idiotype antibody therapy remains an interesting area of investigation, its applicability is limited by patient specificity (ie, antibodies are "tailor-made" for a single patient) and the presence of antibody in the serum of many patients. Recent data indicate that some tumors are biclonal; this would require the use of more than one antibody.[434,435] In addition, the tumor cell idiotype may be unstable due to somatic mutation within the immunoglobulin variable region genes.[436,437]

A number of centers are studying toxin and drug conjugates with murine antibodies directed toward human tumors; clinical trials have just begun. Antisera and monoclonal antibodies conjugated to radionuclides for tumor imaging have been extensively studied; this subject was recently reviewed.[438] We have used the T101 antibody conjugated to ^{111}indium for imaging in 12 patients with cutaneous T cell lymphoma.[439,440] Tumors as small as 0.5 cm have been localized; however, nonspecific uptake of the immunoconjugate in the liver and spleen has prevented critical evaluation of these organs. This difficulty has been partially circumvented by the administration of intracutaneous injections of the immunoconjugate which cause it to be carried via the lymphatics directly to lymph node sites of disease.[441] This procedure does not, of course, facilitate visualization of extralymphatic disease.

Survival for patients with ALL following relapse has not improved over the past several years with chemotherapy drugs. Allogeneic bone marrow transplantation clearly leads to improved survival, but only 30% to 40% of patients have matched donors.[442–444] An alternative method to allogeneic bone marrow transplantation would make use of monoclonal antibodies to cleanse autologous bone marrow prior to bone marrow transplantation. Patients who are in clinical remission are likely to have morphologically undetectable tumor cells in their bone marrow; these cells may be identified and destroyed in vitro by specific antibodies and complement or antibodies conjugated to toxins. In one recently reported study, patients with ALL in second or subsequent remission had their bone marrow treated with a mixture of the BA-1, BA-2, and BA-3 monoclonal antibodies and rabbit complement.[445] All the patients were prepared for transplantation with cyclophosphamide and fractionated total body irradiation. Engraftment occurred in all the patients and 7 of the 23 patients were relapse-free from 6 to 32 months (median 21 months) posttransplantation. All but one of the deaths was caused by recurrent leukemia. The researchers concluded that autologous bone marrow transplantation using in vitro-treated marrow was safe, allowed engraftment, and resulted in prolonged survival in some patients with ALL in second or subsequent remission. Similar results have been reported for ALL patients treated with the J5 monoclonal antibody and complement.[446] Relapse of leukemia in these patients may result from the inadequacy of the preparative regimen used to treat the patients prior to transplantation, inadequate removal by the in vitro treatment with monoclonal antibody and complement, or possibly to the lack of the putative graft *v* leukemia effect described in allogeneic bone marrow transplantation.[447] Even in allogeneic transplantation, in which the preparative regimens are identical to those of autologous transplantation, >50% of the ALL patients relapse, suggesting that an insufficient preparative regimen may be the factor leading to relapse in autologous transplantation as well.

In another study, patients with advanced B cell non-Hodgkin's lymphoma underwent in vitro bone marrow treatment with the anti-B1 antibody and complement.[448] Ten of 17 patients are disease-free at a median follow-up of 22 months (L.M. Nadler, personal communication). Despite the presence of the B1 antigen on mature B cells, B cells recovered within the first few months after transplantation, suggesting that the normal B cell progenitor does not express the B1 antigen.

Another approach to cleansing bone marrow in vitro is the use of monoclonal antibodies conjugated to toxins. In one study, whole ricin was conjugated to the T101 and 3A1 antibodies.[449] It was demonstrated that 95% of the tumor colonies were killed whereas 96% of bone marrow progenitor cells survived. Similar results were reported for a panel of anti-T cell monoclonal antibodies conjugated to intact ricin.[450] Other investigators reported results of an immunotoxin synthesized with pokeweed antiviral protein and the B43 antibody directed against Burkitt lymphoma cells.[451] Immunotoxins may prove to have advantages over antibody and complement; not all antibodies fix complement, and immunotoxins may have greater cytotoxic capability.

The use of monoclonal antibodies and antibody immunoconjugates in the treatment and radioimaging of cancer is in its infancy. Although much work must still be done to address the problems of monoclonal antibody therapies, studies in animal tumor models and humans have clearly demonstrated that antibodies alone or antibody conjugates can be safely administered with minimal adverse effects; in selected cases, these may have diagnostic and therapeutic value. Nonspecific localization of antibody in the reticuloendothelial system, host antibody response, and antigenic heterogeneity are major obstacles to safe and effective treatment with monoclonal antibodies. These issues are under investigation in animal models and humans. Although anti-idiotype antibodies are highly specific and have produced excellent responses in a small number of patients, problems such as biclonality of some lymphomas, instability of the idiotype, and the difficulty of tailoring antibodies to individual patients clearly limit the role of anti-idiotype therapy. The utility of purging bone marrow in vitro with antibodies and complement (or antibodies coupled to toxins) is limited to only a few diseases. However, studies have demonstrated that tumor cells can be removed from the bone marrow following in vitro treatment with antibody and complement; treated bone marrow can successfully engraft, and a number of patients have remained disease-free for >2 years. Whether this is related to the in vitro treatment is unknown. This treatment may prove to be an important application of monoclonal antibody therapy, and it bypasses most of the problems associated with in vivo monoclonal antibody serotherapy. Perhaps the most important future role for monoclonal antibody therapy will be in patients with minimal disease in the "adjuvant" setting, in whom antibody conjugates may eliminate micrometastatic deposits of tumor cells. This remains to be addressed in controlled trials.

CONCLUSION

The application of hybridoma technology and the exciting discoveries in molecular biology over the past 10 years have led to major advances in our understanding of the cellular origin of leukemia and lymphoma and will likely lead to a better understanding of the etiology of these diseases. Utilizing these techniques, it is now possible to more accurately diagnose and classify these disorders, sometimes guiding therapeutic decisions. It is also possible to use molecular probes to detect minimal residual disease. In the future, monoclonal antibodies conjugated to isotopes, drugs, and/or toxins will likely have a role in the therapy of certain leukemias and lymphomas. We look forward to this exciting new era in cancer therapy and diagnosis.

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Dye-Mediated Photolysis of Human Neuroblastoma Cells: Implications for Autologous Bone Marrow Transplantation

By Fritz Sieber, Sanjay Rao, Scott D. Rowley, and Maya Sieber-Blum

Cells from three different human neuroblastoma cell lines and normal human bone marrow cells were exposed to the lipophilic fluorescent dye, merocyanine 540 (MC 540), and white light. In vitro clonogenic tumor cells were inactivated up to 25,000 times more rapidly than multipotent hematopoietic progenitor cells (CFU-GEMM). It is conceivable that this pronounced difference in sensitivity to MC 540-mediated photolysis can be exploited for the selective killing of residual neuroblastoma cells in autologous remission marrow grafts.

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BONE MARROW transplantation allows escalation of antineoplastic therapy without regard to marrow toxicity. It thus affords a greater chance of eradicating tumors.[1,2] Autotransplantation of the patient's own cryopreserved remission marrow virtually eliminates one of the most serious complications of allogeneic marrow transplantation, graft-*v*-host disease (GVHD). It also obviates the need for HLA-identical allogeneic marrow donors.[3-7] However, if the tumor originated in or metastasized to the bone marrow, transplantation of autologous remission marrow carries a significant risk of reinfusing occult tumor cells.[8,9]

For many tumors, the minimal concentration of neoplastic cells required for their unequivocal detection in bone marrow is ~1%. Thus, in the worst situation, a clinical marrow graft could contain between 10^8 and 10^9 residual tumor cells and still be classified as a remission marrow graft. Much effort is therefore currently directed at developing more sensitive methods for the detection of residual tumor cells and methods for their selective elimination from autologous remission marrow grafts.[10-17]

Studies with experimental tumors have shown that most physical, pharmacologic, and immunologic purging procedures are capable of reducing the concentration of tumor cells by one to four (occasionally six) orders of magnitude. We may thus have to resort to combination purging protocols to process marrow grafts with high concentrations of residual tumor cells. A combination of methods that are based on different principles may be the most promising strategy.

We have recently shown that Neuro 2a and NB41A3 murine neuroblastoma cells are killed very rapidly when they are exposed to the lipophilic photosensitizer, merocyanine 540 (MC 540) and a strong source of white light.[17] By contrast, normal murine pluripotent hematopoietic stem cells are only minimally damaged by the same treatment. Lethally irradiated mice that are transplanted with photosensitized mixtures of tumor cells and normal marrow cells survive and remain free of disease.[17] In this article, we present evidence that the same approach may also be applicable to the purging of human marrow grafts contaminated with neuroblastoma cells. MC 540-mediated photolysis reduced the concentration of in vitro clonogenic human neuroblastoma cells 1,000-fold to 50,000-fold, yet preserved 55% of multipotent hematopoietic progenitor cells (CFU-GEMM) from normal bone marrow.

MATERIALS AND METHODS

Cells. The human neuroblastoma cell lines IMR-32 (CCL 127), SK-N-MC (HTB 10), and SK-N-SH (HTB 11) were obtained from the American Type Culture Collection (Rockville, Md) and maintained in a culture medium consisting of 10% heat-inactivated fetal bovine serum (FBS) (KC Biological, Lenexa, Kan) and 90% α-modified minimal essential medium (α-MEM) (α-medium; KC Biological). Aliquots from a frozen stock of cells were thawed at regular intervals to prevent the unintentional selection of mutants. All three lines were free of mycoplasma infections.

The IMR-32 line originates from an abdominal mass in a 13-month-old child with a tumor diagnosed as neuroblastoma with rare areas of organoid differentiation.[18] The line expresses adrenergic traits and consists of two morphologically distinct cell types, small neuroblast-like cells and large hyaline fibroblasts. The SK-N-MC line was isolated from a metastasis to the supraorbital area in a 14-year-old child.[19] The cells have epithelial-like morphology and express cholinergic traits. Surface marker patterns are consistent with those of a neuroepithelioma.[20] The SK-N-SH line was isolated from a bone marrow metastasis in a 4-year-old child.[19] The cells are epithelial-like with adrenergic characteristics. The IMR-32 line was established before treatment was initiated. The two other lines were established after the patients had received radiotherapy and chemotherapy.

Normal human bone marrow cells were obtained from healthy donors for clinical bone marrow transplants after informed consent had been obtained under a protocol approved by the Joint Committee on Human Investigation and in accord with an assurance filed with and approved by the Department of Health and Human Services. A mononuclear cell fraction was prepared by centrifugation on Ficoll-Paque (Pharmacia, Piscataway, NJ).[21]

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Dye-mediated photolysis. Single-cell suspensions of neuroblastoma cells were prepared by incubating subconfluent monolayers briefly with trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA/L Hanks' balanced saline; GIBCO, Grand Island, NY) and filtering the detached cells through a nylon screen (HD3-15; Tetko, Elmford, NY) to remove cell aggregates. The filtered cells were washed once with HEPES-buffered (10 mmol/L, pH 7.4) α-medium supplemented with 5% FBS and resuspended at a density of 1×10^6/mL in HEPES-buffered α-medium supplemented with 15% FBS, penicillin (10 U/mL), and streptomycin (10 μg/mL). Cell viability by trypan blue exclusion was $\geq$98%. Mononuclear marrow cells were suspended in the same medium at a concentration of 5×10^6/mL. MC 540 (Eastman, Rochester, NY) was added from a 1 mg/mL stock solution in 50% ethanol to a final concentration of 15 μg/mL. Clear polystyrene tubes (15-mm diameter, Corning, Corning, NY) containing the cell suspensions were placed into the center of a circular array (40-cm diameter) of 10 fluorescent light bulbs (Champion F15T8/D, 61 cm long). The cell suspensions were swirled manually every 10 minutes to prevent the cells from settling. Samples were removed at 15-minute intervals to determine the surviving fraction of in vitro clonogenic cells. The photosensitization process was stopped by washing the cells twice with HEPES-buffered α-medium supplemented with 5% FBS and antibiotics. This and all subsequent manipulations were carried out in the dark or under subdued lighting.

Bioassays. CFU-GEMM from normal human marrow were assayed as described by Messner and Fauser.[22] Each 35-mm Petri dish contained 10^5 nucleated marrow cells and 1 mL of Iscove's modified Dulbecco's medium (GIBCO) supplemented with 30% FBS bovine serum, 0.9% methylcellulose, 5×10^{-5} mol/L of 2-mercaptoethanol, 1 U of sheep plasma erythropoietin (step III, Connaught Laboratories, Willowdale, Ontario, Canada) and 5% phytohemagglutinin-stimulated leukocyte-conditioned medium. Cultures were incubated for 14 days at 37 °C in a humidified atmosphere of 5% CO_2 in air. Mixed colonies were scored in situ with an inverted microscope. Selected colonies were removed with a finely drawn pipette and stained with benzidine and Wright's-Giemsa to verify their cellular composition.

Two different methods were used to assay in vitro clonogenic neuroblastoma cells.

The first method was used for anchorage-independent cells. Approximately 400 untreated or up to 80,000 photosensitized cells were plated in nonadhesive 35-mm Petri dishes (Greiner, Nuertingen, FRG) containing 1 mL of α-medium supplemented with 20% heat-inactivated FBS and 0.9% methylcellulose (4,000 cPs, Fluka, Buchs, Switzerland) and incubated at 36 °C in a humidified atmosphere of 5% CO_2 in air. Two weeks later, colonies estimated to consist of at least eight cells were scored in situ with an inverted microscope. Plating efficiencies were 32% for IMR-32 and 44% for SK-N-MC cells. This method yielded well-defined, compact colonies that were easy to score even at high densities. It was, however, only applicable to IMR-32 and SK-N-MC cells.

The second method was primarily used for the assay of anchorage-dependent SK-N-SH cells: Approximately 200 untreated or up to 80,000 photosensitized cells were plated in 35-mm tissue culture dishes (Corning) containing 2 mL of α-medium supplemented with 20% heat-inactivated FBS. After 2 weeks at 36 °C in a humidified atmosphere of 5% CO_2 in air, the cultures were fixed by the addition of 0.2 mL of a 25% solution of glutaraldehyde in water. The fixed cells were then stained with Wright's-Giemsa, washed with water, air dried, and evaluated with an inverted microscope. The plating efficiency of SK-N-SH cells was 22%.

SK-N-MC and IMR-32 cells that were routinely assayed in methylcellulose cultures were assayed at least once on adhesive tissue culture dishes to establish that both colony assays yielded equivalent survival curves. Plating efficiencies were lower than in methylcellulose (18% and 31% for IMR-32 and SK-N-MC cells, respectively), but the shape of the survival curves was identical.

Double logarithmic plots of the number of colonies formed as a function of the number of cells plated could be fitted with a straight line with a slope of Δ log (colony count)/Δ log (cells plated) = 1[23], indicating that the colony assays were linear down to limiting dilutions, and that colonies were of clonal origin.

RESULTS

Photosensitization of neuroblastoma and normal bone marrow cells progressively reduced the number but not the size of colonies formed. At the time of evaluation, cultures set up with heavily photosensitized neuroblastoma cells contained few or no cells. However, they contained much cell debris, indicating that MC 540-mediated photosensitization killed and eventually lysed susceptible cells rather than just slowing their growth.

Extensive inhibition of colony formation required exposure to both, dye and light (Table 1). Incubation with dye in the dark had no effect. Exposure to light in the absence of dye reduced the concentration of in vitro clonogenic neuroblastoma cells. However, the reduction was minimal as compared with the reduction achieved by simultaneous exposure to dye and light (Table 1).

All three neuroblastoma cell lines were more sensitive to MC 540-mediated photolysis than were CFU-GEMM from normal bone marrow (Fig 1). Exposure to light for 90

Table 1. Effects of MC 540 and Light on In Vitro Colony Formation by Neuroblastoma Cells

	Cell Line		
Conditions	SK-N-SH	SK-N-MC	IMR-32
Untreated	54.25 ± 2.84	187.25 ± 2.78	135.75 ± 3.66
Light, 90 min	39.50 ± 3.50 (P = .017)	132.00 ± 2.48 (P < .001)	44.25 ± 1.75 (P < .001)
MC 540, dark	53.50 ± 4.66 (P = .895)	214.50 ± 11.08 (P = .054)	137.25 ± 7.73 (P = .867)
MC 540, light, 90 min	0.0425 ± 0.0149 (P < .001)	0.0100 ± 0.0058 (P < .001)	0.0025 ± 0.0025 (P < .001)

MC 540, merocyanine 540.

Data are expressed as mean colony counts of four replicate culture dishes ± SEM. Only simultaneous exposure to both MC 540 and light caused a drastic reduction of colony formation. P values in parentheses: two-tailed Student's t test.

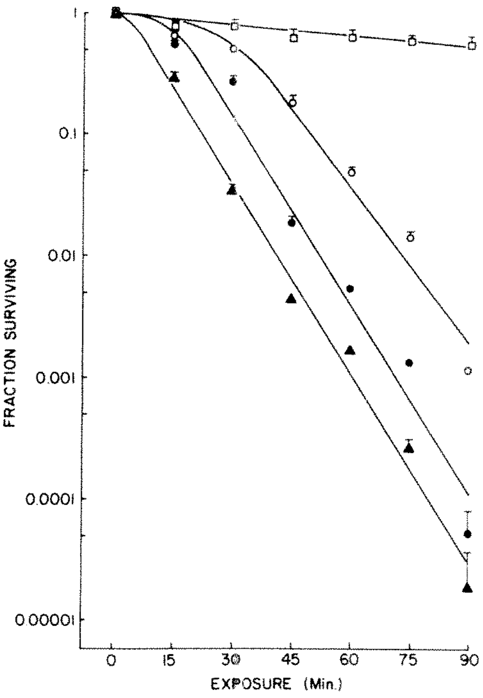

Fig 1. Representative example of the photosensitization of multipotent hematopoietic progenitor cells (CFU-GEMM; □), and three neuroblastoma cell lines, SK-N-SH (○), SK-N-MC (●), and IMR-32 (▲), respectively. All data points represent mean colony counts of four to eight replicate culture dishes. Vertical bars indicate SE. Colony counts at zero time point were 8.7 ± 1.7 (CFU-GEMM), 88 ± 9.4 (SK-N-SH), 201 ± 20.9 (SK-N-MC), and 137 ± 11.2 (IMR-32) respectively. SK-N-SH cells were assayed on adhesive tissue culture dishes; IMR-32 and SK-N-MC cells were assayed in nonadhesive Petri dishes.

minutes reduced the concentration of in vitro clonogenic SK-N-SH, SK-N-MC, and IMR-32 cells 1,000-fold, 10,000-fold, and 50,000-fold, respectively, yet preserved 55% of CFU-GEMM.

Colonies formed by IMR-32, SK-N-MC, and SK-N-SH cells were heterogeneous with respect to size and the morphologic appearance (eg, number and length of neuronal processes) of the cells they contained. A similar degree of heterogeneity was evident in both untreated and photosensitized cultures. Colony heterogeneity was therefore not a result of distinct subpopulations of colony-forming cells that could be distinguished by their sensitivity to MC 540-mediated photosensitization.

When survival curves were plotted semilogarithmically, the portion of the curve to the right of the plateau (Fig 1) could be fitted by a single straight line, indicating that most colony-forming cells of a given neuroblastoma line were about equally sensitive to dye-mediated photosensitization. Subpopulations that were significantly less sensitive than the majority of cells would have resulted in biphasic or multiphasic survival curves. Small subpopulations of photoresistant cells would have been detected most readily in the IMR-32 line where the survival curve spanned >4 logs.

Because the neuroblastoma cells were obtained from cultures in exponential growth phase, they contained cells in all phases of the cell cycle. The linear survival curves thus also indicate that in these three neuroblastoma cell lines all stages of the cell cycle were photosensitive. Again the argument is strongest for the IMR-32 line in which the survival curve spanned >4 logs.

DISCUSSION

MC 540 is an amphipathic fluorescent dye.[24] In the absence of serum, it binds indiscriminately to the plasma membrane of all cells. However, in the presence of serum which acts like a competitive substrate with binding sites of intermediate affinity, the dye binds preferentially to cells with high-affinity binding sites for dye molecules. High-affinity dye-binding sites abound on electrically excitable cells, leukemic cells, and certain classes of immature blood cells.[25-28] Some authors have suggested that MC 540 binds preferentially to disordered[29] or cholesterol-free[30] domains in the outer leaflet of the lipid bilayer. Other authors favor the view that a cell's affinity for MC 540 is a function of the electrical properties of its plasma membrane.[25,26] The phototoxic reaction is not yet fully understood. It probably involves the formation of reactive oxygen species,[24] and plasma membrane lipids may be their primary target (lipid peroxidation). Cells that adsorb large amounts of dye tend to be photolysed more rapidly than are cells that bind small amounts of dye,[31] suggesting that a cell's sensitivity to MC 540-mediated photolysis is primarily a function of the density of high-affinity dye-binding sites.

In this article, we show that three human neuroblastomas were markedly more sensitive to MC 540-mediated photolysis than CFU-GEMM from normal human bone marrow. The fact that the three neuroblastoma lines differed significantly with regard to their sensitivity to MC 540-mediated photolysis is not surprising. The three cell lines were derived from different patients and from primary and secondary tumors from different sites. We do not attach too much importance to the observation that the two lines derived from patients who had undergone chemotherapy and radiotherapy were less photosensitive than the line that had been established from an untreated neuroblastoma. Previous experiments with multiple drug-resistant mutant tumor cell lines have shown that drug-resistant tumor cells were usually not less photosensitive than the corresponding wild type of tumor cell line.[31]

The two cell lines derived from metastatic tumors (SK-N-MC and SK-N-SH) happened to be less photosensitive than the line that originated from a primary tumor (IMR-32). Site-specific alterations in the lipid composition of tumor cells have been described (reviewed in ref. 32). It is conceivable that site-specific differences in photosensitivity occur as well.

Exposure to light in the absence of dye reduced the concentration of in vitro clonogenic neuroblastoma cells by 25% to 65% (Table 1). Similar observations have been made with one human leukemia cell line, murine erythroid progenitor cells, and quail neural crest cells.[33,34] The mechanism of this phototoxic effect is unclear. We have some suggestive evidence that dye-independent phototoxicity is at least in part attributable to the small amount of ultraviolet (UV) light emitted by some white light sources. It is also known that daylight fluorescent light can generate toxic photoproducts in tissue culture medium which can rapidly and irreversibly damage susceptible cells.[35]

Although CFU-GEMM are primitive progenitor cells, they are probably not identical with the pluripotent hematopoietic stem cell. Our data, therefore, do not prove that the human pluripotent hematopoietic stem cell is photoresistant. Such proof could only be derived from the hematologic reconstitution of patients who have received marrow-lethal cytoreductive therapy. However, the striking parallels that exist between the photosensitivities of hematopoietic progenitor cells in mice and humans suggest that the pluripotent hematopoietic stem cell compartment in humans is photoresistant. All classes of progenitor cells that have been tested so far follow the same rank order of sensitivity[33,36-38] in mice and humans. Late erythroid progenitors are the most sensitive cells, followed in order of decreasing sensitivity by early erythroid progenitors, granulocyte/macrophage progenitors, and day 12 spleen colony-forming cells (CFU-S_{12}) (in the mouse) and CFU-GEMM (in humans), respectively. In the mouse, the pluripotent stem cell compartment appears to be even less sensitive than CFU-S_{12},[17,39] because marrow grafts that are essentially depleted of CFU-S_{12} are still capable of rescuing lethally irradiated hosts. By inference, we would therefore expect the human pluripotent hematopoietic stem cell to be at least as photoresistant as CFU-GEMM.

If the high photosensitivity of experimental neuroblastomas can be confirmed in spontaneous tumors, our findings have implications for the purging of autologous remission marrow grafts. Despite our limited understanding of the molecular mechanism of MC 540-mediated photosensitization, it seems reasonable to speculate that the parameters that define a cell's sensitivity to MC 540-mediated photosensitization are quite different from those that define affinity for lectins or antibodies, behavior in density gradients or electrical fields, or susceptibility to most antineoplastic drugs. We, therefore, expect to see little cross-resistance if dye-mediated photosensitization is used in combination with other purging methods. This point may be relevant to the treatment of patients with relapsed neuroblastomas which frequently contain drug-resistant clones.

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Stimulation of Persisting Colonies in Agar Cultures by Sera From Patients With CML and AML

By Ross D. Brown, Edna Yuen, Harry Kronenberg, and Kevin A. Rickard

Cord plasma contains colony-stimulating activity (CSA) which stimulates the in vitro clonal growth of neutrophils, eosinophils, macrophages, erythrocytes, and persisting mast cells in semisolid cultures. Analysis of day 35 colonies in agar cultures was found to be a suitable means of demonstrating this activity and discriminating between it and granulocyte-macrophage colony-stimulating factor (GM-CSF). Serum (10%) from patients with acute and chronic myeloid leukemia (AML and CML) was added to normal human bone marrow cultures to search for similar activity in these patient's serum. Although the number of colonies on day 12 (predominantly neutrophils and macrophages) was not significantly different from the number of colonies in cultures containing normal serum, the number of colonies increased 500% in cultures containing CML serum on day 35. Serum from patients with AML during regeneration also stimulated an increased number of colonies on day 35. Although both eosinophil and mast cell colonies were still present on day 35, only mast cell colonies persisted for 150 days. On day 35, cultures containing 10% CML serum contained predominantly eosinophil colonies (84%), whereas cultures containing AML serum contained predominantly mast cell colonies (76%). Although serum contains various CSFs, the specific factor which stimulates persisting mast cell colonies may be the human equivalent of murine persisting (P) cell-stimulating factor (Multi-CSF).

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THE PRODUCTION and function of granulocytes is controlled by a group of specific glycoproteins known as colony-stimulating factors (CSFs) because of their ability to stimulate hematopoietic precursor cells to form colonies in semisolid medium. Four murine granulocyte-macrophage CSFs (GM-CSF, G-CSF, M-CSF, and Multi-CSF) have been identified and purified.[1-4] cDNAs for murine GM-CSF[5] and murine Multi-CSF, also known as interleukin-3 (IL-3),[6] have been isolated and transfected into cells to produce recombinant CSFs. Purified preparations of both natural and recombinant human GM-CSF have also been produced.[7]

The term persisting (P) cell has been given to a specialized subset of murine mast cells[8] that are capable of prolonged growth in vitro when cultured in the presence of a specific hematopoietic growth factor called P cell–stimulating factor (PSF). PSF is now considered to be identical to a number of other multilineage hematopoietins including burst-promoting activity,[9] IL-3,[4] and Multi-CSF.[10] Murine P cells are mononuclear cells with large metachromatic granules rich in histamine.[9] Variant P cells have arisen that grow in the absence of exogenous PSF by producing autogenous PSF and, unlike the parental P cells, are tumorigenic in vivo.[11] It has been suggested that the autogenous production of PSF may be a mechanism for the malignant transformation of hematopoietic cells.[11,12]

We recently reported the existence of persisting mast cell-like colonies in cultures of human cord blood cells.[13] In this report, we demonstrate that human bone marrow cells in the presence of cord plasma can also grow persisting colonies and that these persisting colonies are the result of colony-stimulating activity (CSA) other then GM-CSF. We also report the relative concentration of this CSA in the serum of patients with acute and chronic myeloid leukemia (AML and CML).

MATERIALS AND METHODS

Sample collection. Bone marrow samples were collected into sterile 5-mL plastic tubes containing 100 IU of heparin after informed consent was obtained from all subjects. Peripheral blood (adult and cord) was collected into 10-mL plastic tubes containing 125 IU of heparin. Plasma was removed after centrifugation and stored at -20 °C.

Agar cultures. Marrow samples were spun at 150 to 200 g for 15 minutes. The plasma and buffy coat were removed and washed in Hanks' balanced salt solution (HBSS) (CSL, Melbourne). Cells were cultured at 2×10^5 cells per milliliter in 0.3% agar medium (McCoys) (GIBCO, Grand Island, NY) as described previously.[14-15] Cultures were stimulated with either 10% giant cell tumor-conditioned medium (GCT-CM) (GIBCO), 10% cord plasma or a combination of two of these factors. Petri dishes were incubated at 37 °C in a humidified atmosphere containing 7.5% CO_2. Agar cultures were kept hydrated for long incubation periods by bubbling air/CO_2 through a 4-L water bath in the bottom of a relatively small incubator (National Model 3221, USA).

Each type of colony had a typical appearance that could be determined with a dissecting microscope (Nikon Model 85615, Tokyo). Confirmation studies were performed on several hundred colonies by removing individual colonies with a Pasteur pipette, squashing them on a slide, and staining them with either May-Grunwald-Giemsa, Astra blue, or the specific histamine stain *o*-phthalaldehyde.[16]

BFU-E assay. The erythroid burst assay was performed as previously described.[17] One-milliliter plasma clot cultures were established in NCTC-109 (GIBCO) with 10% beef embryo extract (GIBCO), 10% bovine serum albumin (BSA) (Sigma, St. Louis), 25% fetal calf serum (FCS) (Flow, Sydney), 15% horse serum (CSL, Melbourne), 10% bovine citrated plasma (Sigma), and 5×10^5 nucleated cells. Cultures with and without 10% cord serum were placed in 35-mm Petri dishes without erythropoietin and incubated for 15 days at 37 °C in a humidified incubator with 7.5% CO_2.

RESULTS

Figure 1A shows a 35-mm Petri dish containing persisting (day 64) colonies in agar cultures stimulated by GCT-CM

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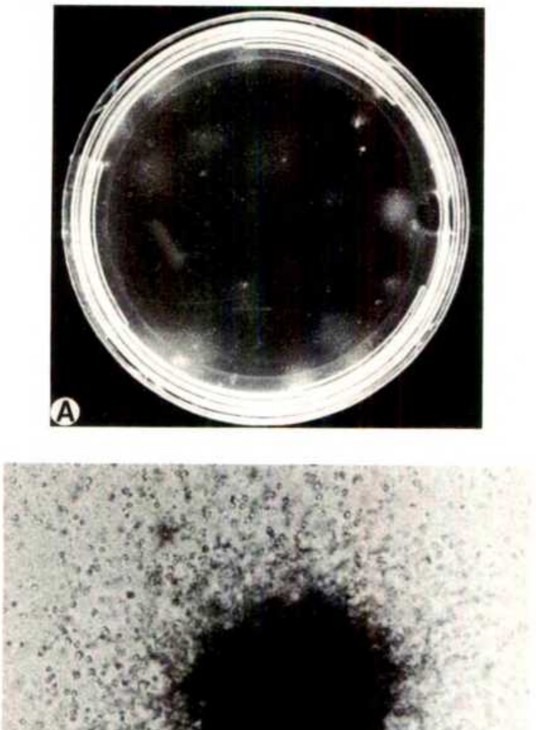

Fig 1. **(A) Petri dish (35 mm) containing persisting colonies stimulated by both GCT-CM and cord plasma on day 64 of culture. (B) Typical day 35 eosinophil colony. (C) Typical day 35 mast cell colony.**

and cord plasma. In cultures of cord blood, colonies grew up to 8 mm in diameter by day 64. Because of the addition of fresh agar medium every month, persisting colonies have continued in vitro for >150 days. Figure 1B shows a typical day 35, dark and compact eosinophil colony; Fig 1C shows a typical, day 35, mast cell colony containing loosely packed, large clear cells. The identity of the mast cells was confirmed by staining with Astra blue and the specific histamine immunofluorescent stain *o*-phthalaldehyde.[16] Mixed colonies of mast cells and eosinophils occurred, but at a very low frequency. Detailed morphologic studies of day 12 neutrophils and day 35 eosinophils and mast cells have been previously demonstrated.[13]

The total number and type of colonies in GCT-CM stimulated normal human marrow cultures with and without 10% cord plasma were determined on both day 12 and 35. A total of 40 different cord plasma samples and 16 different normal marrow samples were studied. Table 1 shows some typical results from four different normal marrows each stimulated by three different cord plasmas. Bone marrows 1 and 2 were both stimulated by the same three cord plasmas, yet produced a different proportion of eosinophil and mast cell colonies. In all our experiments, colonies of mast cells and eosinophils continued to grow in vitro if stimulated by both cord plasma and GCT-CM, whereas neutrophil colonies degenerated and disappeared soon after their peak on day 12. Cord plasma alone stimulated colonies on day 35 only if there was significant marrow autostimulation (eg, BM 3). The dose–response curve for the concentration of cord plasma was sigmoid but linear for the number of cells cultured.

The number of colonies in GCT-CM stimulated normal human bone marrow cultures containing 10% serum from patients with AML ($N = 4$), AML in remission ($N = 9$), and CML ($N = 14$), as compared with normal control sera ($N = 9$) when counted on day 12 is shown in Fig 2. There was a fairly wide spread in the overall results; however, the mean and the range was similar for each group. Eight different normal bone marrow samples were used to test these AML and CML sera.

Figure 3 illustrates the number of colonies in the same cultures as in Fig 2 when recounted on day 35. There was a >500% increase in the mean number of persisting colonies in the cultures (day 35) containing CML serum (mean number of colonies = 25.2) as compared with normal serum (mean number of colonies = 4.8). Serum from patients with AML in early remission also stimulated an increased number of persisting colonies. Cultures with AML or CML sera but without GCT-CM had very few or no colonies on day 35.

The type of cells in day 35 normal marrow cultures containing 10% patient's serum and 10% GCT-CM is illustrated in Fig 4; 84% of the colonies in cultures containing CML serum were eosinophilic, whereas 76% of the colonies in cultures containing nonremission AML sera were mast cell colonies. Although eosinophil colonies were still present on day 35 of these cultures, only mast cell colonies persisted for periods of several months. The cellular integrity of eosinophils in 35-day-old cultures was deteriorating. Consistent eosinophil/mast cell ratios were observed within each group of patients when different marrow samples were used with the same test sera and also when the same marrow was used to test sera from different patient groups.

There was a prolific growth of erythroid bursts in BFU-E cultures of normal bone marrow cells without exogenous erythropoietin but containing 10% cord plasma. In control cultures without cord plasma, virtually no growth occurred.

Table 1. Effect of Cord Plasma on Colony Growth From Four Different Normal Human Marrow Samples

		Number of Colonies per 2 × 10⁵ Marrow Cells				
		Day 12	Day 35			
BM	Stimulus	Total Neut/Mono	Total	Neut/Mono	Mast	Eosin
1.	GCT-CM only	21 ± 3	0	0	0	0
	GCT-CM + CP*	36 ± 3	29 ± 8	0	15 ± 8	14 ± 2
	CP only	3 ± 2	1	0	1	0
	Auto	0	0	0	0	0
2.	GCT-CM only	10 ± 2	0	0	0	0
	GCT-CM + CP*	35 ± 4	20 ± 9	0	18 ± 8	2 ± 1
	CP only	10 ± 4	3 ± 1	0	2 ± 1	1
	Auto	0	0	0	0	0
3.	GCT-CM only	40 ± 5	0	0	0	0
	GCT-CM + CP*	44 ± 3	48 ± 6	0	3 ± 1	45 ± 6
	CP only	32 ± 9	43 ± 9	0	3 ± 1	40 ± 9
	Auto	11 ± 4	3	0	1	2 ± 1
4.	GCT-CM only	18 ± 2	1	0	1	0
	GCT-CM + CP*	18 ± 3	25 ± 13	0	2 ± 1	23 ± 12
	CP only	12 ± 3	1	0	1	0
	Auto	10 ± 2	0	0	0	0

Results are expressed as mean ± SD.

GCT-CM, giant cell tumor-conditioned medium; BM, bone marrow; CP, cord plasma; Auto, autostimulated; Neut, neutrophils; Mono, monocytes; Mast, mast cells; Eosin, eosinophils.

*Mean of 3 plasmas.

DISCUSSION

Normal human bone marrow progenitor cells have been stimulated to produce colonies of eosinophils and mast cells in 35-day-old agar cultures by a CSA that we first described in cultures of cord blood cells.[13] The results have shown that this CSA is not GM-CSF and is present in the serum of patients with CML at a concentration which is, on average, 500% higher than in normal serum. Patients with AML in early remission also tended to have an increased concentration of this CSA. Although the stimulation of these additional colonies may be owing to more than one specific CSF, we suggest that the persisting mast cell colonies may be the human equivalent of the murine P cell,[8] and the CSA involved may be human PSF, otherwise known as IL-3 or Multi-CSF.[4,10] If this suggestion is true, our results indicate that in the serum of patients with CML there is a 500% increase in the concentration of a factor that is known to stimulate stem cell self-renewal. This, together with other factors such as a decreased release of neutrophil-derived lactoferrin,[15] may well cause the characteristic expansion of the myeloid stem cell pool in CML. Future studies should include a biochemical characterization of this CSA and isolation of cDNA clones.

Studies involving the addition of serum from patients with leukemia to normal marrow cultures were performed by several groups more than a decade ago.[18,19] Like those described in the results in Fig 2, these cultures were counted after 12 to 14 days of incubation and thus were detecting GM-CSF and GM-CSF inhibitor levels in leukemia. Neither these previous studies nor our results in Fig 2 produced results of any real significance.

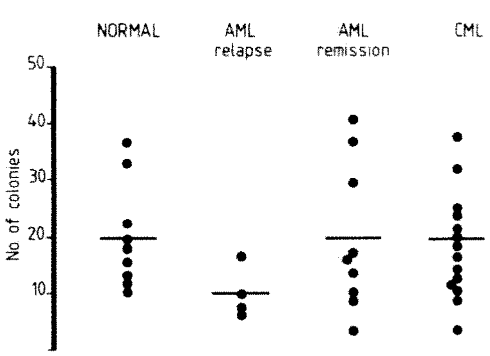

Fig 2. The number of colonies per 2 × 10⁵ cells plated in GCT-CM–stimulated normal human bone marrow cultures containing 10% serum from patients with acute myelogenous leukemia (AML), AML in remission, and chronic myelogenous leukemia (CML) as compared with a normal control group on day 12.

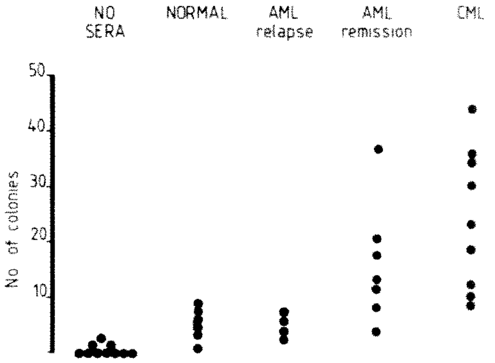

Fig 3. The number of colonies per 2 × 10⁶ cells plated in normal GCT-CM–stimulated cultures containing 10% serum from patients with acute myelogenous leukemia (AML), AML in remission, and chronic myelogenous leukemia (CML) as compared with a normal control group on day 35. The same cultures were counted as in Fig 2.

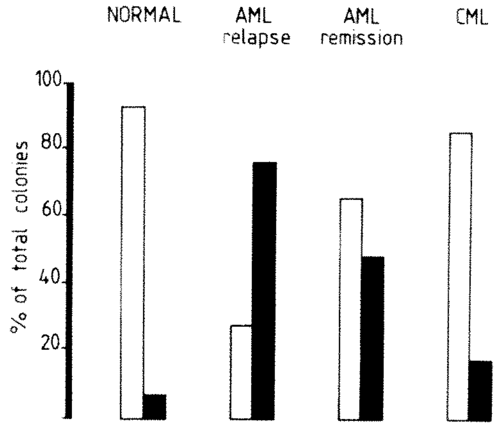

Fig 4. The proportion of eosinophil and mast cell colonies in day 35 normal bone marrow cultures containing 10% serum from patients with acute myelogenous leukemia (AML), AML in remission, and chronic myelogenous leukemia (CML). ☐, Eosinophil; ■, mast.

The fact that day 35 colonies in cultures containing CML sera were predominantly eosinophilic whereas day 35 colonies in cultures containing AML serum were predominantly mast cells is an interesting phenomenon that is difficult to explain. Although the eosinophil/mast cell ratios were found to be relatively consistent for each AML or CML sera tested (Fig 4), there was considerable variation in the eosinophil/mast cell ratio when cord plasma was tested (Table 1). The observations that different cord plasma samples could stimulate the same proportion of eosinophil and mast cell colonies in day 35 cultures of the same normal marrow cells, yet a single cord plasma sample did not necessarily stimulate the same proportion of eosinophil and mast cell colonies when used in cultures of different normal marrow cells (Table 1), suggests that the specific line of differentiation is determined not only by the exogenous stimulating activity but also by other factors, including the target cells within the marrow cell population. Perhaps future studies may reveal that AML and CML have a similar etiology in terms of oncogenesis, with the transforming gene of a tumor-inducing virus infecting stem cells at various levels of differentiation. Thus, subtle differences in gene rearrangements which involve the expression of growth factors may produce various forms of myeloid leukemia.

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Fucose Binding Lectin for Characterizing Acute Myeloid Leukemia Progenitor Cells

By Ruud Delwel, Ivo Touw, Freek Bot, and Bob Löwenberg

The reactivity of acute myeloid leukemia cells (AML) was determined in 29 patients using the fucose binding lectin *Ulex europaeus* agglutinin (UEA) as surface marker. We show a marked heterogeneity in the UEA-binding abilities of the cells in these patients as determined by fluorescence analysis of the blasts labeled with the UEA coupled to the fluorescent molecule FITC. The results suggest a correlation between the capability of AML blast cells to bind UEA and cytologic maturation, because in 1 of 10 M1, 3 of 8 M2, 6 of 8 M4, and 1 of 3 M5 cytology types UEA binding to the leukemic cells was apparent. In 13 cases, the cells gave rise to colonies in vitro. The amount of UEA binding to AML colony-forming cells (AML-CFU) was determined by cell sorting and subsequent colony culture of UEA-negative, intermediately positive, and highly fluorescent cells. AML-CFU from none of the four patients with M1 cytology were UEA positive, whereas they showed intense reactivity with the lectin in 1 of 4 cases with M2 cytology and in all 4 cases of M4. In these five cases with strongly UEA positive AML-CFU, the fluorescence distribution of the colony formers differed from that of the total leukemia population, indicating that AML-CFU represent a subpopulation of AML cells with specific UEA-binding properties. Normal bone marrow myeloid and multipotential colony-forming cells (CFU-GM, CFU-GEMM) showed low or no binding of UEA. UEA-FITC appears a useful reagent for membrane analysis of AML-CFU. In certain cases, UEA-FITC labeling may be applied to discriminate AML-CFU from normal hematopoietic progenitors.

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SEVERAL STUDIES have dealt with the detailed phenotyping of acute myeloid leukemia colony-forming cells (AML-CFU) in relation to normal bone marrow colony formers (CFU-GM, CFU-GEMM).[1-3] The results of those studies indicate that AML-CFU can be recognized as the leukemic counterparts of normal hematopoietic progenitor cells. These investigations were performed with monoclonal antibodies, which have become the most common tools to identify surface phenotypes.[4-14]

Lectins may also be of value to detect surface antigens. For example, the fucose-binding lectin from *Lotus tetragonolobus* (FBL-L) has been shown to react with human myeloid cells.[15-18] The amount of cellular binding of the lectin increases progressively from myeloblasts toward mature granulocytes; monocytes show intermediate reactivity. CFU-GM mainly belong to the low FBL-L–binding bone marrow fraction. No information exists on the usefulness of the lectin for analysis of AML-CFU.

In the present study, we labeled AML blasts with the fucose binding lectin (FBL) *Ulex europaeus* agglutinin coupled to fluorescein-isothiocyanate (UEA-FITC) to determine whether UEA-binding to AML-CFU is different from that of normal CFU-GM and CFU-GEMM. We also compared UEA binding patterns of AML-CFU with that of the total AML blast population and with morphological classification of AML according to the criteria of the French-American-British (FAB) group.[19]

It is shown that: (a) in certain cases, UEA binding to AML-CFU is markedly discrepant from that of normal CFU-GM and CFU-GEMM; (b) binding patterns of AML-CFU can be distinguished from those of total blast populations; and (c) UEA binding capacity of AML-CFU is positively correlated to cytologic (FAB) maturation.

MATERIALS AND METHODS

Cell separation. Cells from 29 patients with newly diagnosed AML and from three subjects without hematological disease were studied. AML cytology was classified according to the FAB criteria.[19] All individuals had given consent to donate bone marrow. AML blasts from bone marrow or peripheral blood were separated to >97% purity following discontinuous bovine serum albumin (BSA) density fractionation and E rosette sedimentation.[20-22] The cells were then cryopreserved in aliquots of 30 × 10^6 cells in 7.5% dimethyl sulfoxide (DMSO) and 20% fetal calf serum (FCS) using a controlled freezing apparatus and storage in liquid nitrogen as described.[23] Normal bone marrow cells were recovered from the interface after Ficoll-Isopaque centrifugation.[24]

Colony cultures for AML-CFU, CFU-GM, and CFU-GEMM. Colonies from AML cells were cultured in the PHA-leukocyte feeder (PHA-LF) system.[20,21,25] Colonies in this system from purified blasts are always of leukemic nature as indicated by morphology, immunophenotypes,[5] and cytogenetic markers.[20,21,25] Myeloid colonies (CFU-GM) from normal marrow were grown in a semisolid culture containing human placenta conditioned medium (HPCM; 20% vol/vol) as a source of colony-stimulating factor (CSF).[26,27]

Mixed colonies (CFU-GEMM) were grown as described by Fauser and Messner with slight modifications.[28] Normal bone marrow cells were cultured in a 1-mL mixture of Iscove's modified Dulbecco's medium (IMDM, GIBCO, Gent, Belgium), 1.1% methylcellulose, 30% autologous heparinized plasma, 7.5% of a medium conditioned by leukocytes in the presence of 1% PHA (PHA-LCM), 1 U sheep erythropoietin per milliliter (step III, Connaught, Willowdale, Ontario, Canada), BSA, transferrin, lecithin, sodium selenite, and 2-mercapto-ethanol. Dishes were incubated at 37 °C and 100% humidity in an environment of 5% CO_2 in air. Colonies were scored at day 15. Mixed colonies were defined as containing red hemoglobinized cells plus at least 5% nonhemoglobinized translucent cells of various sizes. Disputable mixed colonies were always verified cytologically after they were lifted off the plate with a fine-drawn Pasteur pipette.

Labeling of cells with UEA-FITC and FACS. AML blast cells or bone marrow nucleated cells were suspended in phosphate-buffered saline (PBS) containing 5% FCS at a cell concentration of

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2×10^7 cells/mL. The suspension was incubated for 30 minutes at 4 °C with 20 μL of UEA-FITC (Polysciences Inc, Warrington, Pa). The cells were then washed three times with PBS and resuspended in PBS with 5% FCS at a cell concentration of 2×10^6 cells per milliliter. Cell sorting was performed on a FACS 440 (Becton Dickinson, Sunnyvale, Calif). Excitation was performed with the laser at 488 nm (0.4 W). For the fluorescence measurements, a 530/30 band pass filter was used. The instrument was calibrated with fluorescent standard beads (1.0 μm and 2.83 μm in diameter) (Polysciences Inc).

Dead cells among the leukemic population were excluded from analysis by elevating the threshold level of the forward light scatter. Fractions of different fluorescence intensity were sorted following a sterile procedure and plated separately in colony culture for determining the number of colony-forming cells as a function of the amount of UEA binding. Usually, between 1.5 to 3.0×10^5 cells per fraction were collected. Sorting rate was between 1,500 and 2,000 cells/second. Data analysis was performed using a Hewlett Packard 86B system.

May-Grünwald-Giemsa staining. After FACS, normal bone marrow cells or peripheral blood cells were suspended in 50% FCS in PBS (4×10^5 cells in 0.1 mL) and centrifuged onto clean glass slides (500 rpm for 5 minutes). The slides were air dried and, after fixation in methanol (1½ minutes), they were stained with May-Grünwald's solution for 3 minutes and then counterstained with 4% (vol/vol) Giemsa in PBS. Slides were then washed and air dried. Differential counts were performed at ×1,000 magnification under oil, counting 250 cells.

RESULTS

UEA-binding to AML-blasts and AML progenitors. The reactivity of purified AML cells to the fluorescent FBL (UEA-FITC) was studied. In 11 of 29 cases, the AML blasts reacted with the lectin (Table 1). The data are indicative of a correlation between the binding of UEA and morphologic maturation of AML cells according to the FAB classification. The blasts of only 1 of 10 patients with AML-M1 cytology were UEA reactive, whereas in 3 of 8 cases of AML-M2, 6 of 8 AML-M4, and 1 of 3 AML M5, the cells showed significant UEA binding.

Of the complete series of patients, in 13 cases the cells were capable of giving rise to colonies in vitro. To determine the quantitative level of surface binding of UEA-FITC to AML-CFU, cells were sorted into separate fractions of varying fluorescence intensity and seeded into PHA-LF. AML-CFU from subjects with a relatively undifferentiated AML morphology, ie, all four M1 cases, showed no or minimal capacity for UEA-binding (Table 2), whereas AML precursor cells from one of four patients with M2 and four of four patients with M4 cytology exhibited high UEA binding. The colony formers of the single patient with acute monoblast leukemia (M5) did not bind detectable UEA to their surface. In Fig 1, four typical examples are given of the fluorescence distribution profiles of AML blast cells and AML-CFU. The cells from three patients also formed colonies in the other culture system, ie, with HPCM as stimulator. The distributions of AML-CFU as assessed in these cultures were found to be identical to those of AML-CFU grown in the PHA-LF method (data not shown).

UEA binding to normal bone marrow cells, CFU-GM, and CFU-GEMM. For comparison, the UEA-binding properties of normal marrow cells, CFU-GM and CFU-GEMM were determined. Figure 1 gives an example of the UEA-FITC fluorescence distribution of Ficoll-separated normal marrow cells and CFU-GM. CFU-GEMM, not plotted, showed essentially identical UEA binding properties as CFU-GM. It is evident from this and the data from the separate experiments shown in Table 2 that low amounts of UEA-FITC bind to the cell surface of normal CFU-GM and CFU-GEMM.

Table 1. Binding of UEA-FITC in Relation to FAB Classification of AML

Classification*	UEA Negative	UEA Positive†
M1 (n = 10)	9	1
M2 (n = 8)	5	3
M4 (n = 8)	2	6
M5 (n = 3)	2	1
Total N = 29	18	11

UEA, *Ulex europaeus* agglutinin; AML, acute myelogenous leukemia.

*Morphologic subtypes based on the classification of the French-American-British (FAB) Cooperative Group.[19]

†Of the 11 UEA-positive cases, 100% of the cells were UEA reactive in 7 patients (1 M1, 2 M2, 4 M4), and 15% to 20% of the cells reacted with the lectin in the other 4 cases (1 M2, 2 M4, 1 M5).

Table 2. Distribution of Nucleated Cells, AML-CFU, CFU-GM, and CFU-GEMM Relative to the Amount of UEA Binding

		UEA Negative (−)†		UEA Weakly Positive (+)‡		UEA Strongly Positive (++)§	
Patient	FAB*	Nucleated Cells (%)	CFU (%)	Nucleated Cells (%)	CFU (%)	Nucleated Cells (%)	CFU (%)
1	M1	100	100	0	0	0	0
2	M1	95	100	4	0	1	0
3	M1	97	100	2	0	1	0
4	M1	97	100	2	0	1	0
5	M2	91	100	8	0	1	0
6	M2‖	26	54	53	46	21	0
7	M2	94	100	6	0	0	0
8	M2	39	18	27	26	34	56
9	M4	22	10	45	18	33	72
10	M4	24	8	21	29	55	63
11	M4	13	0	33	20	54	80
12	M4	49	5	33	33	18	63
13	M5	98	100	2	0	0	0
Normal BM CFU-GM¶		64	51	16	46	14	3
Normal BM CFU-GM#		54	26	36	66	15	8
Normal BM CFU-GM		49	44	30	52	21	4
Normal BM CFU-GEMM		49	13	30	80	21	7
Normal BM CFU-GEMM		62	29	17	57	21	14

Abbreviations as in Table 1. BM, bone marrow.

Percentage of total recovered (%).

*Classification according to the FAB nomenclature.[19]

†Negative fluorescence (log scale); channel 0–90.

‡Weakly positive fluorescence; channel 91–130.

§Strongly positive fluorescence; channel 131–255.

‖Channel numbers of sorted fractions: (0–90), (91–126), (127–255).

¶Channel numbers of sorted fractions: (0–90), (91–142), (143–255).

#Channel numbers of sorted fractions: (0–90), (91–147), (148–255).

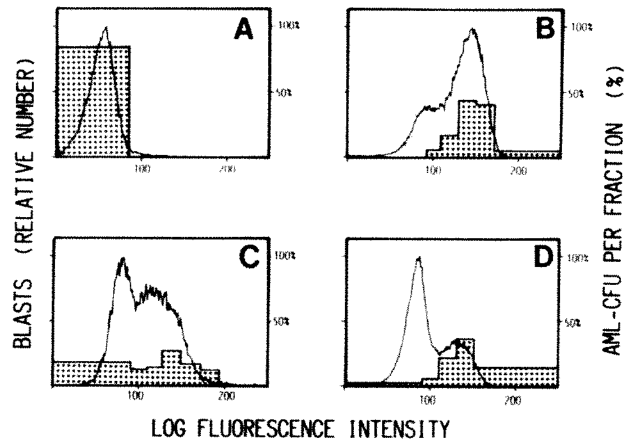

Fig 1. Distribution of acute myelogenous leukemia (AML) blast cells (——) and AML-CFU (▨) as a function of the fluorescence intensity following *Ulex europaeus* agglutinin (UEA)–FITC labeling (four representative profiles). Fractions: 0 through 90; 91 through 110; 111 through 130; 131 through 150; 151 through 170; and 171 through 255. (A) Patient 4 (FAB-M1): AML blasts and AML-CFU are UEA negative. (B) Patient 11 (FAB-M4): heterogeneous quantities of UEA are bound to AML blasts. AML-CFU are recovered from the blast cell fraction, intensively reactive with UEA. (C) Patient 8 (FAB-M2): broad peak of UEA binding indicating cellular heterogeneity. Most AML-CFU are UEA reactive. (D) Patient 12 (FAB-M4): most AML blasts are weakly UEA positive, but most AML-CFU are selectively recovered from the strongly UEA-positive subpopulation.

In addition, the relative density of UEA binding of different marrow cells was determined following cell sorting and subsequent differential counting of the smear (Table 3). Nucleated red cells were highly reactive with the lectin, whereas >50% of the monocytes showed intermediate to strong UEA binding. Only part of the promyelocytes/myelocytes were weakly UEA positive. All other cell types were found in the negative fraction. Peripheral blood monocytes and most of the eosinophils showed high UEA reactivity whereas neutrophilic granulocytes were intermediately positive for UEA. Basophilic granulocytes were equally distributed; lymphocytes were UEA negative.

Table 3. UEA-FITC Binding of Normal Bone Marrow Cells

Fraction* (Cell Type)	Percentage of Recovery of Different Cell Types as a Function of UEA-Binding Intensity							
	1	2	3	4	5	6	7	Total
Myeloblasts	70	27	0	0	0	3	0	100 (6)†
Promyelocytes, myelocytes	59	37	1	1	1	1	0	100 (13)
Metamyelocytes, polymorphs	93	5	1	0	1	0	0	100 (10)
Monocytes	40	10	16	15	10	6	3	100 (8)
Nucleated red cells	0	0	1	0	1	10	88	100 (<1)
Lymphocytes	97	3	0	0	0	0	0	100 (64)

UEA, *Ulex europaeus* agglutinin.

*Ficoll-separated bone marrow nucleated cells were sorted into fractions on the basis of *Ulex europaeus* (UEA)-FITC fluorescence intensity. Channel numbers of sorted fractions: fraction 1 (0–90, negative), 2 (91–110), 3 (111–130), 4 (131–150), 5 (151–170), 6 (171–190), and 7 (191–255, strongly positive).

†Total of each cell type was set at 100%. Recoveries of different cell types from Ficoll-separated marrow are indicated in parentheses.

DISCUSSION

In this study, we examined the reactivity of the UEA to AML blast cells and AML-CFU. The findings were related to the maturation stage of AML (FAB) and to UEA reactivity of normal bone marrow nucleated cells, CFU-GM and CFU-GEMM. Lectin binding to the surface of AML blast cells among the patients (N = 29) showed considerable variability. The cell samples from 18 patients were UEA negative; the blasts from 11 patients were UEA positive. Fluorescence histograms indicate that UEA reactivity is heterogeneously distributed among blasts, indicating subsets with relatively low and high UEA surface binding.

Although the number of cases was limited, a correlation is suggested as regards the capacity of the cells to bind UEA and their morphology. The M1 cases with one exception were negative, whereas cells from three of eight M2 cases and six of eight M4 cases reacted with the lectin. The cells from one of three cases of an acute monoblastic leukemia (M5), showed UEA reactivity. The data are compatible with the results from investigations with FBL-L.[28] In the latter studies, it was demonstrated that 5 of 6 M4 cases and the 1 M2 case reacted with this FBL-L, whereas their 1 M5 case was FBL-L negative. No AML cells classified as M1 were examined in that particular study. FBL-L and UEA are both reactive with α-L fucose. UEA can be washed from the cells with this sugar (data not shown). The reactivity of the two lectins, however, differs somewhat. FBL-L has a higher binding capacity for granulocytes than for monocytes.[16–18] Our results show the opposite for UEA, ie, intense binding to monocytes and an intermediate binding to neutrophilic granulocytes. Studies in larger numbers of patients appear of interest in order to evaluate the usefulness of UEA as a reagent in the classification of AML.

The fluorescence histograms shown in Table 1 reveal three discrete types of reactivity among the leukemias: a group of cases of fully UEA-negative AML (N = 18), a second group of AML containing ~20% UEA-positive cells (N = 4) and a

Table 4. UEA-FITC Binding to Peripheral Blood Cells

Fraction* (Cell Type)	Percentage of Recovery of Different Cell Types as a Function of UEA Binding Intensity					
	1	2	3	4	5	Total
Neutrophils	13	44	25	7	11	100 (87)†
Basophils	2	2	13	27	56	100 (6)
Eosinophils	15	14	19	20	32	100 (1)
Monocytes	1	4	21	31	43	100 (1)
Lymphocytes	86	7	1	2	4	100 (5)

UEA, *Ulex europaeus* agglutinin.

*Methylcellulose sedimented peripheral blood nucleated cells were sorted into fractions on the basis of UEA-FITC fluorescence intensity. Channel numbers of sorted fractions: fraction 1 (0–90, negative), 2 (91–110), 3 (111–130), 4 (131–150), and 5 (151–255, strongly positive).

†Total of each cell type was set at 100%. Recoveries of different cell types from methylcellulose sedimented blood are indicated in parentheses.

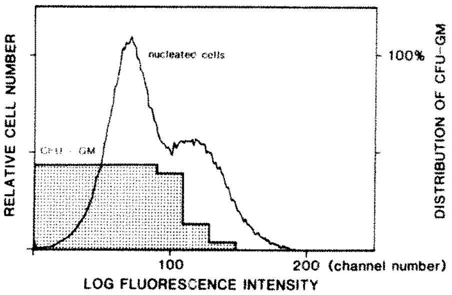

Fig 2. Distribution of normal bone marrow nucleated cells and CFU-GM as a function of fluorescence intensity following *Ulex europaeus* agglutinin (UEA)-FITC labeling. Fractions: 0 through 90; 91 through 110; 111 through 130; 131 through 150; and 151 through 255.

third series of seven cases, in which 100% of the cells reacted with the lectin. Because we had no sufficient cell material to study colony growth among cases of each of these three groups, we were not able to correlate these classes to colony-forming abilities or AML-CFU phenotypes. However, it appeared that of the fully UEA-positive AML cases with colony growth, the majority of the AML-CFU were recovered from the UEA weakly positive fraction in one case and, in the other 5 cases, from the UEA strongly positive fraction.

Sorting experiments using UEA-FITC fluorescence show that in several cases AML-CFU can be recognized as a subgroup of cells with specific lectin-binding properties. This is consistent with the results of immunophenotyping, which have indicated that AML-CFU are frequently different from the total AML population in their antigenic cell surface profile.[9,12,13]

The relation between UEA binding and FAB categories holds up when binding is expressed for the AML-CFU subpopulation. In 4 M1 cases, UEA binding to AML-CFU was low (Table 2), whereas in 1 of 4 M2 and 4 of 4 M4 patients, a high level of UEA binding sites was demonstrated on the cell membrane. Variations in the expression of membrane antigens of AML-CFU have been noticed by different groups.[12-14] Correlations between immunophenotypes of AML-CFU and AML cytology have been established but have provided controversial results. On the basis of the antigenic cell surface structure, Lange and co-workers classified AML-CFU as counterparts of the in vitro multipotential stem cell (CFU-GEMM), "early" CFU-GM or "late" CFU-GM. These investigators did not find a correlation with FAB classification of AML cells.[1] However, using another panel of monoclonal antibodies, Sabbath and colleagues concluded that the antigenic makeup of AML-CFU tended to correlate with the maturation stage of the cells.[2] We demonstrate that CFU-GM and CFU-GEMM were generally UEA negative or weakly positive. We suggest that AML-CFU with little UEA reactivity are generally of a more immature phenotype, whereas AML-CFU with high binding capacities are more differentiated into monocytic directions.

Morstyn and colleagues have shown that early as well as late CFU-GM have low affinity for FBL-L.[18] In our studies, AML-CFU in 5 patients (1 AML-M2, 4 AML-M4) showed high reactivity with the lectin UEA and, in 8 cases, AML-CFU were recovered from the UEA low-reactive or negative fractions. It is possible that the discrepant UEA-binding capacities of AML-CFU and normal colony formers can be exploited in certain cases for recognizing AML clonogenic cells in the bone marrow during complete remission.

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Effects of Purified Bacterially Synthesized Murine Multi-CSF (IL-3) on Hematopoiesis in Normal Adult Mice

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Normal adult C57BL, BALB/c, and C3H/HeJ mice were injected intraperitoneally three times daily for up to 6 days with 102,000 U (200 ng) per injection of purified, bacterially synthesized, Multipotential colony-stimulating factor (CSF) (Interleukin-3) (rMulti-CSF) and compared with control mice injected with serum/saline with or without added endotoxin (1 ng/mL). Mice injected with rMulti-CSF exhibited tenfold rises in blood eosinophil and twofold to threefold rises in neutrophil and monocyte levels. The spleens from mice injected with rMulti-CSF showed a 50% increase in weight, elevated levels of maturing granulocytes, eosinophils, nucleated erythroid cells and megakaryocytes, and up to 100-fold rises in mast cells. Progenitor cell frequencies in the spleen were elevated sixfold to 18-fold. No significant changes were observed in the marrow. Sixfold to 15-fold rises were observed in peritoneal cell populations of mice injected with rMulti-CSF with evidence of increased peritoneal macrophage phagocytic activity. Livers of C57BL mice, but not of the other strains, exhibited increased numbers of infiltrating hematopoietic cells whereas rises in mast cell numbers were observed in the mesenteric lymph node, skin, and gut in BALB/c and C3H/HeJ mice. Endotoxin was excluded as being responsible for the observed changes except possibly those involving peritoneal macrophage phagocytic activity. The results indicate that the injection of normal mice with rMulti-CSF significantly stimulates the same types of hematopoietic populations as are stimulated in vitro by Multi-CSF and indicate that this and other CSFs should be useful in stimulating hematopoietic repopulation and functional activity in vivo.

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IN VITRO STUDIES have documented the existence of a series of specific regulatory glycoproteins able to control the proliferation and functional activity of various murine hematopoietic subpopulations.[1] Prominent among these is the glycoprotein, multipotential colony-stimulating factor (Multi-CSF), also known as interleukin-3 (IL-3),[2,3] hemopoietic cell growth factor (HCGF),[4] burst-promoting activity (BPA),[5] or persisting cell-stimulating factor (PSF).[6] This molecule has been purified to homogeneity either from medium conditioned by WEHI-3B myelomonocytic leukemic cells[2,6] or lectin-stimulated spleen T lymphocytes.[7] The purified molecule has been shown to stimulate: (a) some self-generation by hematopoietic stem cells with, in addition, the formation of various progenitor cells; (b) the clonal proliferation in semisolid cultures of multipotential, granulocytic, macrophage, eosinophil, erythroid, and megakaryocytic progenitor cells to form colonies of differentiating progeny; (c) the proliferation of mast cells; and (d) the survival and proliferation of various continuous hematopoietic cell lines.[1-7]

Studies by two groups resulted in the initial isolation of cDNA clones for Multi-CSF and deduction of the full amino acid sequence of the polypeptide portion of the molecule.[8,9] Expression of biologically active Multi-CSF (IL-3) has been achieved using either monkey COS cells[8-10] or a bacterial expression system.[11] The recombinant Multi-CSF produced in both types of expression system was found to exhibit the various biological activites noted for the purified native molecule[8-13] (D. Metcalf and G.R. Johnson, unpublished data, March 1985).

The broad proliferative actions of Multi-CSF revealed by in vitro studies suggest that this molecule should have many demonstrable effects on hematopoiesis when injected in vivo. However, the minute amounts of native Multi-CSF available from purification of Multi-CSF–containing media have to date prevented any attempt to establish the in vivo effects of injected native Multi-CSF. With the demonstration that bacterially synthesized Multi-CSF exhibits the in vitro biological actions of the native molecule, it has become reasonable to use this recombinant material for initial studies to determine the effects of injected Multi-CSF on hemopoietic populations in vivo.

In the present study, purified, bacterially synthesized, Multi-CSF was injected for up to 6 days into normal adult mice. The results indicated that injected Multi-CSF of this type is able to induce various changes in hematopoietic tissues, in general agreement with expectations from the known actions of Multi-CSF in vitro.

MATERIALS AND METHODS

Bacterially-synthesized Multi-CSF. The recombinant Multi-CSF (rMulti-CSF) used in these studies was a generous gift from Dr J. DeLamarter (Biogen, SA, Geneva). The *E coli* expression system used to generate this rMulti-CSF (IL-3) and the steps used to purify the bacterially synthesized material have been described elsewhere.[11,14] The bacterially synthesized material was subjected to final purification by reverse-phase high-performance liquid chromatography (HPLC) using a Brownlee RP300 (25 cm, 7 mm ID) column. The rMulti-CSF was recovered using an increasing gradient of acetonitrile (1%/min). The linear gradient was formed at 1 mL/min over 60 minutes with aqueous trifluoroacetic acid (0.15% vol/vol) as a primary solvent and acetonitrile (60% vol/vol) in trifluoroacetic acid (0.1% vol/vol) as a secondary solvent, using a Beckman liquid chromatograph. A single major peak of optical density was observed, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the peak fraction demonstrated

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the presence of a single silver-staining band with a mol wt of 16,000. The peak fraction (1 mL) from the HPLC column was diluted in 10% C57BL mouse serum/saline and passed through a Sephadex G-25M (PD-10, Pharmacia, Uppsala, Sweden) column to remove residual acetonitrile.

After passage through the PD-10 column, the fraction containing rMulti-CSF was diluted in 10% C57BL mouse serum/saline to a final concentration of 200 ng rMulti-CSF per 0.2 ml (102,000 U/0.2 mL).

Material for injection into control mice was saline containing 10% C57BL serum or this material with added endotoxin (lipopolysaccharide W, *E coli* 0111:B4) Difco Laboratories, Detroit) (final concentration, 0.25 ng/mL or 1 ng/mL in serum/saline).

Tests for endotoxin levels in the rMulti-CSF and control preparations used for injection were performed using the Limulus assay[15] (Limulus amebocyte lysate, Whittaker MA Bioproducts, Walkersville, Md) prior to, and following, each series of injections (Table 1).

Mice. Mice used (3-month-old male and female C57BL/6fJ/WEHI, BALB/c/An/Bradley/WEHI or C3H/HeJ mice maintained in our institute) were matched for sex and body weight in each experiment. Mice were reared under specific pathogen-free conditions and then were held in conventional animal rooms for 6 weeks before use.

Injection schedule. Mice were injected intraperitoneally (IP) with 0.2 mL of rMulti-CSF (102,000 U per injection) or 0.2 mL of serum/saline or serum/saline containing endotoxin. Injections were performed three times daily at 0800, 1600, and 2200 hours. All analyses were performed commencing at 0900, the last injection being at 2200 the preceding evening.

WBC and hematocrit estimations. Mice were anesthetized with ether and orbital plexus blood obtained using 50-μL micropipettes. No mouse was examined on more than one occasion to avoid misleading cell counts from previously damaged and/or inflamed vessels. WBC counts were performed using hemocytometers and differential cell counts performed on blood films stained with May Grünwald-Giemsa. Hematocrit estimations were performed using microhematocrit tubes and reticulocyte counts performed on blood films stained with Cresyl blue.

Examination of tissues. After white cell counts were performed, mice were reanesthetized with ether, and the peripheral blood was collected from the axillary vessels into heparinized tubes. Following death of the mice from exsanguination, 2 mL of saline was injected IP into each mouse and, after gentle massage of the abdominal cavity, the peritoneal wall was opened and as much fluid as possible was collected with a sterile pipette.

Bone marrow cells were collected from one intact femur shaft, and the spleen was removed aseptically. Portions of the following tissues were taken for histological examination after fixation in 10% formalin in saline: spleen, kidney, liver, mesenteric node, small bowel adjacent to the mesenteric node, lung, heart, skin, and thymus. All sections were prepared in duplicate and stained with hematoxylin and eosin or, for mast cell enumeration, with Alcian-blue-safronin. Cytocentrifuge preparations were made of peritoneal, bone marrow, and spleen cell suspensions and stained with May Grünwald-Giemsa.

Bioassay of rMulti-CSF levels. Levels of rMulti-CSF in injected material, serum, or peritoneal fluid were assayed in serial twofold dilutions in 1 mL agar-medium cultures[1] using either 75,000 C57BL bone marrow cells or 300 32D cells, selectively responsive only to stimulation by Multi-CSF.[16] Cultures were incubated for 7 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Colony formation (clones > 50 cells) was scored at ×35 using a dissection microscope. Units of CSF per milliliter were calculated from the linear portion of the dose–response curve, 50 U being the concentration stimulating the formation of half-maximal numbers of colonies.

Assay of progenitor cell levels in injected mice. Dispersed cell suspensions were prepared from femoral marrow, a portion of the spleen, and a portion of the peritoneal cavity cells. Assays were performed in 1-mL agar cultures in 35-mm Petri dishes using 50,000 spleen or peritoneal cells or 25,000 marrow cells per culture. The medium used was Dulbecco's modified Eagle's medium (DMEM) with a final concentration of 20% fetal calf serum (FCS) and 0.3% agar.[1] Colony formation was stimulated by addition of 3,200 U of GM-CSF semipurified from mouse lung conditioned medium.[17] Cultures were incubated for 7 days in a fully humidified atmosphere of 10% CO_2 in air, and colony formation (clones > 50 cells) was scored at ×35 using a dissection microscope.

For CFU-E, BFU-E and mixed-erythroid CFC assays, 100,000 spleen or 50,000 bone marrow cells were cultured in 1-mL cultures containing 0.9% methylcellulose (Fluka, Buchs, Switzerland), Iscove's modified Dulbecco's medium (GIBCO, NY) 60 μg/mL of penicillin, 100 μg/mL streptomycin, 5×10^{-5} mol/L of 2-mercaptoethanol, and 20% vol/vol FCS. Erythroid colony formation was stimulated by the addition of 1.6 U human urinary erythropoietin (specific activity 10 to 2,000 U/mg)[18] for CFU-E-derived colonies and 1.6 U erythropoietin plus 0.1 mL of pokeweed mitogen-stimulated spleen-conditioned medium[19] for BFU-E and Mix-CFC colony formation.

Quantitative estimation of tissue content of megakaryocytes and mast cells. Camera lucida drawings of the spleen, mesenteric node, skin, and gut sections were traced on bond paper at ×12 magnifications, and the organ outline was cut out and weighed. Most of the organ drawings were between 100 and 350 mg. After total megakaryocytes or mast cells in the entire section were counted, the comparative number of cells per organ was determined by the following calculation: number of cells times organ weight in milligrams per drawing weight in milligrams or, in the case of skin and gut, number of cells times 100 per drawing weight in milligrams.

Phagocytosis assays using blood neutrophils and peritoneal cells. Heparinized blood was pooled from the four mice in each experimental group, as were harvested peritoneal cells. Blood neutrophils were enriched by centrifugation of the heparinized blood on a two-layer discontinuous density gradient of hypertonic Metrizamide (Nyegaard A/C, Oslo). Blood was mixed with an equal volume (1 mL) of medium containing 10 U/mL of heparin and loaded onto a gradient containing 2 mL of 18.5% and 2 mL of 20% Metrizamide.

Table 1. Assay for Endotoxin in rMulti-CSF Preparation Used for Injection

Material Assayed	Coagulation With Dilution of						
	1:1	1:2	1:4	1:8	1:16	1:32	1:64
rMulti-CSF 100,000 U/0.2 mL in serum/saline	—	—	—	—	—		
Serum/saline	—	—	—	—	—		
Standard endotoxin 1 ng/mL	+	+	+	+	±	—	—
Standard endotoxin 1 ng/mL in serum/saline	+	+	+	+	+	—	—

rMulti-CSF, recombinant multipotential colony-stimulating factor.

Assays performed using Limulus amebocyte lysate assay[15] and were performed on material remaining in bottles after completion of the injection schedule for mice injected with rMulti-CSF or serum/saline. The endotoxin standard was added to the same batch of mouse serum/saline used for injection; + = coagulated and ± = markedly increased viscosity. Mixture of rMulti-CSF with standard endotoxin did not inhibit the ability of endotoxin to be detected.

The cells were then centrifuged for 30 minutes at 1,200 g. The second interface contained 70% to 90% neutrophils, 5% to 10% eosinophils, and 10% to 30% mononuclear cells. Peritoneal cells were obtained as described above.

The phagocytosis assay used sheep erythrocytes coated with antibody (EA) as indicator cells and was carried out as previously described,[20] with minor modifications. The medium used throughout was RPMI 1640 containing 20 mmol/L of HEPES and 2% FCS. Erythrocytes were coated with antibody by incubating 5 × 10^7 cells in 1 mL with 10 μL (saturating amount) of ascites containing a mouse monoclonal IgG2b anti-E antibody[21] (Commonwealth Serum Laboratories, Parkville, Victoria, Australia) or a mouse monoclonal IgG2b anti-DNP (dinitrophenyl) antibody (K1), a gift from Dr C. Sanderson, N.I.M.R., London. Before incubation with the latter antibody, erythrocytes were coupled with TNP (trinitrophenyl) as described previously.[22] Erythrocytes were incubated with antibody for 40 minutes at 22 °C, after which the EA were washed three times to remove unbound antibody. Equal volumes (150 μL) of peritoneal cells or blood neutrophils (10^6/mL) and antibody-coated erythrocytes (5 × 10^7/mL) were then mixed in serology tubes and brought into contact by gentle centrifugation for 20 seconds at 100 g. After this, the mixtures were incubated in a water bath at 37 °C for 10 minutes (this short incubation time was necessary to avoid the lysis and disappearance of many phagocytosed erythrocytes). The incubation was stopped by adding 5 mL of ice-cold medium to the cells and washing them at 4 °C. From this stage on, the cells were kept on ice. The supernatants were then aspirated, and the cells were resuspended in 200 μL of ice-cold distilled water for 10 seconds to lyse extracellular erythrocytes. Isotonicity was quickly restored by adding 5 mL of ice-cold medium. The mixtures were then washed at 4 °C, the pellets were resuspended in a small volume of medium, and cytocentrifuge preparations were made. After the cells were fixed, they were stained with Giemsa. For each cell pool, incubations were performed in triplicate or quadruplicate, and at least 200 cells were counted per slide. Counts were performed on the percentage of phagocytosing cells and the total number of phagocytosed erythrocytes per 100 cells.

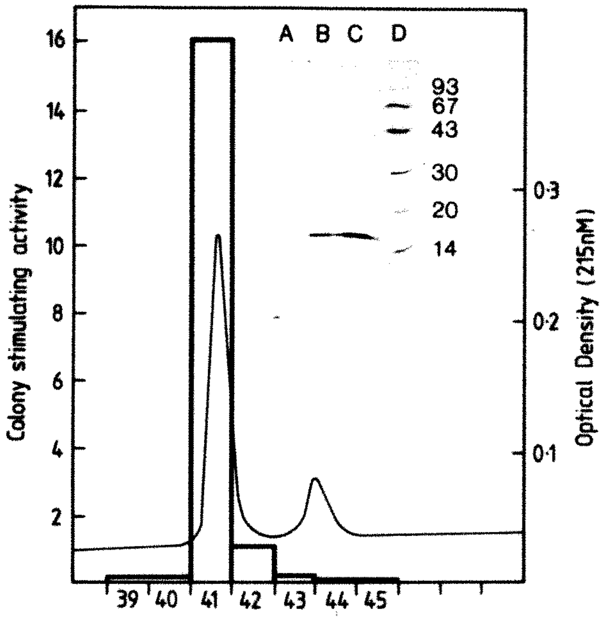

Fig 1. Final purification of bacterially synthesized recombinant multipotential colony-stimulating factor (rMulti-CSF) using reverse-phase high-performance liquid chromotography (HPLC). Assays for biological activity were performed using cultures of C57BL bone marrow cells. Insert shows the polyacrylamide gel electrophoresis of the peak fraction that exhibits a single silver-staining band of apparent mol wt 16,000. (A through C) increasing concentrations of peak fraction. (D) Mol wt markers: phosphorylase b, 93,000; bovine serum albumin 67,000; ovalbumin 43,000; carbonic anhydrase 30,000, soybean trypsin inhibitor 20,000; lysozyme 14,000.

RESULTS

When the recombinant Multi-CSF purified from *E coli* was subjected to final purification using HPLC reverse-phase fractionation, biological activity was coincident with a single ultraviolet (UV)-adsorbing peak (Fig 1). Material in the peak fraction ran as a single silver-staining band of mol wt 16,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Based on granulocyte-macrophage colony formation in bone marrow assays and amino acid analysis of the peak fraction, the biological activity of the purified recombinant Multi-CSF was found to be 5 × 10^8 U per milligram. This material was diluted 1:500 in 10% mouse serum/saline after passage through a PD-10 column to remove residual acetonitrile. The volume of rMulti-CSF injected was 0.2 mL per injection and contained 102,000 U of rMulti-CSF (200 ng).

Toxicity studies on material injected. Assays for endotoxin content were performed on each batch of recombinant Multi-CSF and the carrier serum/saline both prior to and following each course of injection, sampling the material actually used for injection. No endotoxin was detected in any batch used and, as shown in the example in Table 1, the Limulus amebocyte lysate test used was able to detect 0.06 ng of endotoxin per milliliter, detection not being prevented by addition of mouse serum/saline or recombinant Multi-CSF. Despite the absence of detectable endotoxin, in some studies control mice were injected with mouse serum/saline to which 0.25 ng or 1.0 ng of endotoxin had been added per milliliter (that is up to 16 times the endotoxin level that might possibly have been present in the Multi-CSF preparations). In view of the essentially negative results obtained with these mice, only the data from mice injected with 1.0 ng of endotoxin per milliliter will be shown.

In preliminary studies, injection of mice with recombinant Multi-CSF still containing the acetonitrile in trifluoroacetic acid used for elution from the HPLC column was observed to lead to the development of lipid accumulation in the liver. Because of this, all material used in the studies to be described was processed through Sephadex G-25 M (PD-10) columns to remove the solvent, and no lipid accumulation was noted in the liver of mice injected with the processed material. As an additional control study, in four separate experiments acetonitrile in trifluoroacetic acid at the same concentration present in the Multi-CSF preparation prior to PD-10 processing was added to serum/saline. Injection of this material to mice induced lipid accumulation in the liver but failed to induce any of the hematopoietic changes to be described in mice injected with Multi-CSF (peritoneal cell changes, infiltration of cells in the liver, spleen size increase, changes in spleen cell content or progenitor cell levels).

Assays of rMulti-CSF levels achieved by injection. To determine the levels of rMulti-CSF achieved by a single IP injection of 102,000 U, groups of adult C57BL mice were injected, then serum was collected at intervals from 10 minutes to 6 hours. After killing at each timepoint, 2 mL of 10% FCS/saline was injected IP and, after abdominal massage, a sample of the fluid was withdrawn. For purposes of calculation, it was assumed that the resident volume of fluid in the peritoneal cavity was 0.2 mL. Assays for rMulti-CSF levels were performed on serial twofold dilutions of serum or harvested peritoneal fluid using agar cultures of 75,000 C57BL marrow cells. To verify that the CSF detected was indeed Multi-CSF, parallel assays were performed using agar cultures of 32D cells, responsive only to stimulation by Multi-CSF.[16] The data from the two sets of assays were in close agreement, and those from the bone marrow assays are shown in Fig 2, presented as the concentration of rMulti-CSF in BM U/mL of serum or per milliliter of the assumed resident volume of peritoneal fluid (0.2 mL). rMulti-CSF levels in the peritoneal cavity fell, with a half-life (t½) of ~20 min. Concentrations in excess of 50 U/mL (the concentration stimulating the formation of half-maximal numbers of colonies in an agar culture) were maintained for 4 hours. Serum levels of Multi-CSF rose sharply following injection and achieved peak levels between 0.5 and 1 hour following injection. Peak levels were ~6000 U/mL and thereafter levels fell again with a t½ of ~20 minutes. Serum levels above 50 U/mL were sustained for ~2.5 hours.

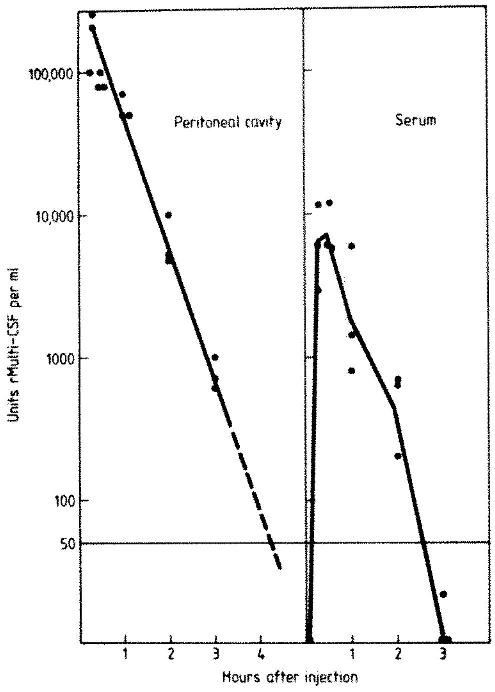

Fig 2. Levels of recombinant multipotentential colony-stimulating factor (rMulti-CSF) in the serum and peritoneal cavity in U/mL at intervals after a single intraperitoneal injection of 102,000 U of bacterially synthesized rMulti-CSF. Assays performed using serial dilutions of test material in cultures of 75,000 C57BL bone marrow cells. Each point represents an individual mouse.

It was concluded that each intraperitoneal injection achieved concentrations of rMulti-CSF that would be supramaximal in in vitro colony assays (ie, >400 U/mL) for 2 hours in the serum and 3 hours in the peritoneal cavity and significantly elevated levels (ie, >50 U/mL) for 2.5 hours in the serum and 4 hours in the peritoneal cavity.

White cell levels. As shown in Fig 3, C57BL mice injected three times daily with rMulti-CSF developed up to a tenfold rise in blood eosinophil levels as compared with control mice injected with serum/saline. Eosinophil levels rose progressively as injections were continued. Minor and less constant rises (twofold to threefold) were observed in polymorph and monocyte levels. No significant changes were observed in lymphocyte, reticulocyte, or hematocrit levels during or immediately after a 6-day course of injections. No mast cells, megakaryocytes, or nucleated red cells were observed in the peripheral blood of mice injected with rMulti-CSF.

Spleen changes. Spleens from C57BL mice injected for 6 days with rMulti-CSF showed a consistent weight increase of 50% (Table 2). Analysis of cytocentrifuge preparations of spleen cell suspensions (Table 2) showed a fourfold rise in the percentage of promyelocytes with primary granules and myelocytes and a twofold to threefold rise in blast cells, eosinophils, and nucleated red cells (the latter including recognizable erythroblasts), as compared to those of uninjected mice or control mice injected with serum/saline.

In histological sections of the spleens of mice injected with rMulti-CSF, no obvious changes were observed in lymphoid follicles or germinal centers. Similarly, there was no overt increase in the content of erythroid or granulocytic cells in the red pulp. However, counts of the numbers of the mature

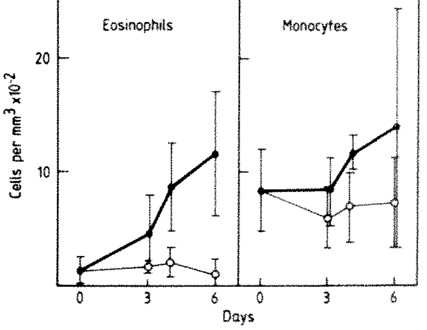

Fig 3. Changes in peripheral blood levels of WBCs in C57BL mice injected with recombinant multipotential colony-stimulating factor (rMulti-CSF) (heavy lines) and in control mice injected with serum/saline (light lines). Each point represents the mean value from 4 to 12 mice ± SD.

Table 2. Changes in Spleen Cell Populations in C57BL Mice Injected With rMulti-CSF

Group	Mean Spleen Weight (mg)	Percentage of Cells						
		Blasts	Promyelocytes and Myelocytes	Metamyelocytes and Neutrophils	Lymphocytes	Monocytes	Eosinophils	Nucleated RBCs
rMulti-CSF	160 ± 70	1.6 ± 1.4	2.8 ± 3.9	9.9 ± 7.3	68.0 ± 16.5	6.3 ± 2.1	1.3 ± 1.1	10.1 ± 5.9
Serum/saline	100 ± 40	0.6 ± 0.8	0.7 ± 1.2	9.3 ± 4.8	80.1 ± 7.7	4.5 ± 2.4	0.6 ± 0.9	4.2 ± 3.2
Uninjected	100 ± 40	0.7 ± 0.8	0.5 ± 0.7	8.9 ± 5.3	81.6 ± 6.6	5.1 ± 2.1	0.3 ± 0.7	2.9 ± 2.3

rMulti-CSF, recombinant multipotential colony-stimulating factor.

C57BL mice injected three times daily for 6 days and examined on day 7. Mean data for 12 mice per group ± SD.

megakaryocytes revealed a twofold to fourfold increase in mice injected for 6 days with rMulti-CSF (Table 3). More dramatic changes were observed in the mast cell content of the spleens of mice injected with rMulti-CSF. In control mice, small numbers of large, heavily granulated mast cells were observed (Fig 4). In the spleens of mice injected with rMulti-CSF, the number of such cells showed no change or a twofold to fourfold increase. However, the spleens of mice injected with rMulti-CSF also contained large numbers of small, incompletely granulated mast cells, which often occurred in clusters reminiscent of in vitro colonies (Fig 4) and were commonly located in the perifollicular sinus regions. The rise in the frequency of these small mast cells was only fivefold to 20-fold in C57BL mice; in C3H/HeJ and BALB/c mice, however, the rise was 100-fold (Table 3). Injection of endotoxin (1 ng/mL) failed to elicit these rises in mast cell numbers.

Spleens from C57BL mice injected for 6 days with rMulti-CSF showed a fivefold to eightfold increase in the frequency of granulocyte and/or macrophage and eosinophil progenitor cells as compared with those of uninjected mice or mice injected with serum/saline (Table 4). Analysis of these colonies revealed no significant differences between the groups in the relative frequency of different colony types. Changes of similar magnitude were noted in the spleens of C3H/HeJ and BALB/c mice following a 6-day injection course. Sequential studies indicated that the elevation in spleen progenitor cell levels in C57BL mice injected with rMulti-CSF was already present after 3 days of injections (rMulti-CSF mice: 44 ± 36 CFC/10^5 cells *v* 8 ± 6 CFC/10^5 cells for serum/saline-injected mice).

Similar changes were observed in levels of erythroid progenitor cells (CFU-E, BFU-E, and Mix-CFC). As shown in Table 5, mice injected for 6 days with rMulti-CSF showed a sixfold to 18-fold rise in CFU-E levels in the spleen and a twofold to tenfold fold rise in the frequency of BFU-E and Mix-CFC.

In three experiments using C57BL, BALB/c, and C3H/HeJ mice, the injection of endotoxin (1 ng/mL) for 6 days failed to elevate progenitor cell levels in the spleen above levels in uninjected mice.

Bone marrow changes. Total cell counts were slightly reduced in the femurs of C57BL mice injected with rMulti-CSF (Table 6). Slight but nonsignificant rises were observed

Table 3. Changes in Megakaryocyte and Mast Cell Numbers in the Spleen of Mice Injected With rMulti-CSF

Strain	Group	Comparative Megakaryocyte Nos. per Spleen	Comparative Mast Cell Nos. per Spleen	
			Large	Small
C57BL				
	rMulti-CSF	479 ± 260	4 ± 3	50 ± 33
	Serum/saline	103 ± 40	4 ± 1	2 ± 1
	Uninjected	70 ± 47	2 ± 3	10 ± 19
C57BL				
	rMulti-CSF	529 ± 462	3 ± 2	94 ± 80
	Serum/saline	259 ± 228	4 ± 3	11 ± 11
	Endotoxin 1 ng/mL	72 ± 40	2 ± 1	1 ± 1
	Uninjected	126 ± 69	7 ± 5	4 ± 4
BALB/c				
	rMulti-CSF	714 ± 135	49 ± 10	1,413 ± 218
	Serum/saline	245 ± 67	16 ± 11	11 ± 6
	Endotoxin 1 ng/mL	277 ± 124	12 ± 9	27 ± 15
	Uninjected	343 ± 77	7 ± 4	12 ± 13
C3H/HeJ	rMulti-CSF	560 ± 107	39 ± 21	1,298 ± 375
	Serum/saline	116 ± 65	14 ± 6	15 ± 4
	Endotoxin 1ng/mL	133 ± 27	11 ± 5	10 ± 5
	Uninjected	230 ± 54	19 ± 17	10 ± 9

rMulti-CSF, recombinant multipotential colony-stimulating factor.

Four mice per group were injected three times daily for 6 days and examined on day 7. Megakaryocyte counts were performed on sections of the entire spleen stained with hematoxylin-eosin. Mast cell counts were performed on sections of the spleen stained with Alcian blue-safronin.

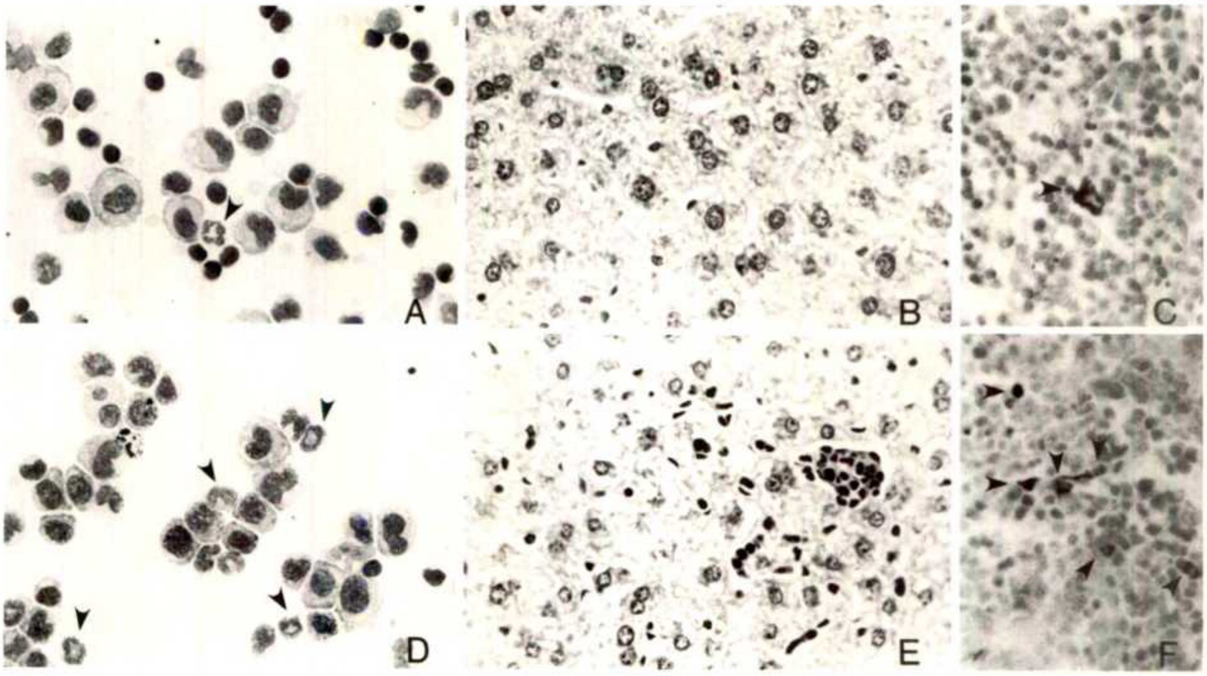

Fig 4. Comparison of mice injected for 6 days with recombinant multipotential colony-stimulating factor (rMulti-CSF) with control mice injected with serum/saline. (A) Control peritoneal cells with a single neutrophil (D). rMulti-CSF peritoneal cells showing increased proportions of macrophages and eosinophils (arrows). (B) Control liver and (E) rMulti-CSF liver showing focal and diffuse infiltration of hemopoietic cells. (C) Control spleen with a solitary large mast cell (arrow). (F) rMulti-CSF spleen showing part of a focus of small, incompletely granulated mast cells (arrows). May-Grunwald-Giemsa stain (A and D); hematoxylin and eosin stain (B and E); Alcian blue-safronin stain (C and F).

in the frequency of blast cells, differentiating granulocytes, and eosinophils. These rises were accompanied by a fall in the frequency of marrow lymphocytes. The frequency of nucleated erythroid cells was not elevated in marrow of mice injected with rMulti-CSF. No mast cells or basophils were observed in the marrow of rMulti-CSF-injected or control mice.

The frequency of granulocyte-macrophage, eosinophil, and erythroid progenitors was essentially similar in the rMulti-CSF-injected and control groups, and no differences were observed in the relative frequencies of colonies of these types (Tables 5 and 7). A similar lack of change in progenitor cell levels was observed in the mice injected for only 3 or 4 days with rMulti-CSF.

Table 4. Elevated Frequency of Progenitor Cells in the Spleens of C57BL Mice Injected With rMulti-CSF

Group	Nonerythroid Progenitors per 10^5 Cells	Percentage of Nonerythroid Colonies G	GM	M	Eosin
rMulti-CSF	48 ± 30	46	27	23	4
Serum/saline	9 ± 4	39	25	32	4
Uninjected	6 ± 7	20	20	60	0

rMulti-CSF, recombinant multipotential colony-stimulating factor; G, granulocytic; GM, granulocyte-macrophage; M, macrophage; Eosin, eosinophil.

Mice were injected three times daily for 6 days and analyzed on day 7. Mean data for 12 C57BL mice in each group ± SD. Replicate cultures of 50,000 spleen cells were stimulated by 3,200 U/mL GM-CSF. At 7 days, colony counts were performed, and colony morphology was determined from whole-culture preparations stained with Luxol Fast Blue-hematoxylin.

Liver changes. Livers of C57BL mice injected for 6 days with rMulti-CSF showed an increased cellular infiltration of two types: (a) a uniformly dispersed infiltration, and (b) in many mice, small focal aggregates of hematopoietic cells not associated with major blood vessels (in many mice) (Fig 4). As shown in Fig 5, counts on random high-power fields that avoided areas of focal infiltration showed an ~50% increase in nonparenchymatal cells per field as compared with those of control mice injected with serum/saline or serum/saline containing 1 ng/mL endotoxin or uninjected mice. The additional infiltrating cells were a mixture of granulocytes and monocytes with a prominent component of eosinophils; the cells in focal aggregates were of the same types. The infiltrating cells were at varying stages of maturation, but mitotic activity was not observed. Occasional megakaryocytes were observed in livers from rMulti-CSF-injected mice but not in control livers. No mast cells or nucleated erythroid cells were observed in any of the livers.

Because of the presence of focal collections of hematopoietic cells in the livers of C57BL mice injected with rMulti-CSF, the above data underestimate the actual levels of infiltrating cells in this strain.

Cellular infiltration was much less apparent in the livers of C3H/HeJ or BALB/c mice injected with rMulti-CSF, as

Table 5. Effect of rMulti-CSF on Frequency of Erythroid Progenitor Cells in Spleen and Bone Marrow

		Erythroid Progenitors per 10^5 Spleen Cells			Erythroid Progenitors per 10^5 Bone Marrow Cells		
Strain	Group	CFU-E	BFU-E	Mix-CFC	CFU-E	BFU-E	Mix-CFC
C57BL Day 4	rMulti-CSF	60 ± 27	26 ± 12	10 ± 9	27 ± 3	6 ± 2	5 ± 2
	Serum/saline	32 ± 29	18 ± 9	5 ± 2	30 ± 16	5 ± 1	4 ± 1
	Uninjected	32 ± 24	6 ± 5	2 ± 1	32 ± 13	3 ± 1	3 ± 2
C3H/HeJ Day 7	rMulti-CSF	314 ± 29	10 ± 4	12 ± 4	97 ± 31	4 ± 2	9 ± 7
	Serum/saline	17 ± 11	1 ± 1	1 ± 1	146 ± 34	3 ± 1	14 ± 8
	Endotoxin 1 ng/mL	46 ± 45	2 ± 1	4 ± 2	78 ± 25	4 ± 2	16 ± 5
	Uninjected	49 ± 18	3 ± 1	5 ± 2	60 ± 20	4 ± 2	21 ± 13

rMulti-CSF, recombinant multipotential colony-stimulating factor.

Groups of four C57BL mice were injected three times daily for 3 days and analyzed on day 4. Groups of four C3H/HeJ mice were injected for 6 days and examined on day 7. Cultures were performed using 10^5 spleen cells or 0.5 × 10^5 bone marrow cells and were scored on day 2 for CFU-E and day 7 for BFU-E and Mix-CFC. Mean data ± SD.

Table 6. Changes in Bone Marrow Populations in C57BL Mice Injected With rMulti-CSF

		Percentage of Cells						
Group	Total Femur Cell Count × 10^{-6}	Blasts	Promyelocytes and Myelocytes	Metamyelocytes and Neutrophils	Lymphocytes	Monocytes	Eosinophils	Nucleated RBCs
rMulti-CSF	21.1 ± 6.2	4.2 ± 1.8	13.3 ± 3.3	49.4 ± 9.3	8.1 ± 4.2	9.0 ± 3.3	6.2 ± 2.8	9.8 ± 5.4
Serum/saline	27.1 ± 4.9	2.8 ± 1.3	12.4 ± 3.8	42.8 ± 8.0	19.8 ± 5.2	7.9 ± 2.6	2.4 ± 1.4	11.9 ± 4.6
Uninjected	25.3 ± 7.6	2.9 ± 1.6	8.0 ± 3.6	42.3 ± 5.2	20.6 ± 4.6	8.1 ± 3.6	4.5 ± 1.6	13.6 ± 5.3

rMulti-CSF, recombinant multipotential colony-stimulating factor.

C57BL mice were injected three time daily for 6 days and then examined on day 7. Mean data for 12 mice per group ± SD.

confirmed by counts on random fields (nonparenchymatal cells per field: C3H/HeJ:rMulti-CSF 10 ± 1, serum/saline 8 ± 2, uninjected 8 ± 1; BALB/c rMulti-CSF 11 ± 1, serum/saline 9 ± 1, uninjected 8 ± 1).

Peritoneal population changes. The most prominent absolute change in cell populations observed in mice injected with rMulti-CSF was an increase in total cells in the peritoneal cavity. The increased cellularity was apparent to the naked eye as a milky opalescence of the harvested cell suspension. As shown in Fig 6, the changes induced by rMulti-CSF in C57BL mice were a sixfold rise in total macrophages, a 15-fold rise in eosinophils and a sevenfold rise in mature neutrophils. Total mast cell and lymphocyte numbers were not altered by rMulti-CSF injections, and no nucleated erythroid cells were observed in the peritoneal cell populations.

Table 7. Frequency of Progenitor Cells in the Bone Marrow of Mice Injected With rMulti-CSF

		Percentage of Colonies			
Group	Nonerythroid Progenitors per 10^5 Cells	G	GM	M	Eosin
rMulti-CSF	141 ± 50	33	40	27	0
Serum/saline	132 ± 44	24	31	45	0
Uninjected	146 ± 50	27	36	30	7

rMulti-CSF, recombinant multipotential colony-stimulating factor.

C57BL mice were injected three times daily for 6 days and then analyzed on day 7. Mean data for 12 mice per group ± SD in replicate cultures of 25,000 marrow cells stimulated by 3,200 U/mL GM-CSF. Colony counts were performed at day 7, and colony morphology was determined from whole-culture preparations stained with Luxol Fast Blue-hematoxylin.

Sequential observations indicated that no increase in peritoneal cell numbers had occurred 3 or 6 hours after a single injection of rMulti-CSF. Neither had the cellular composition of the peritoneal populations changed. The rises in peritoneal cell numbers were progressive during the 7-day observation period.

The additional macrophages appearing in the peritoneal cavity of rMulti-CSF-injected mice tended to be more baso-

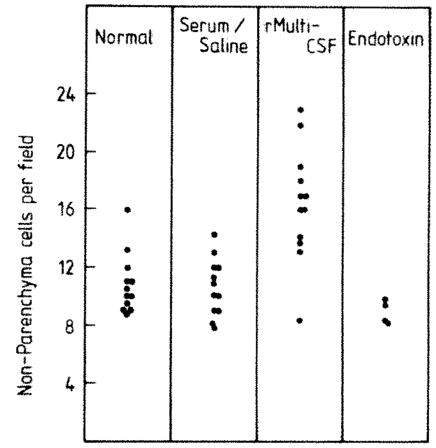

Fig 5. Rise in nonparenchymal cells (granulocytes, macrophages, and eosinophils) per high power field in sections of the liver of C57BL mice injected for 6 days with recombinant multipotential colony-stimulating factor (rMulti-CSF) as compared with the frequency of such cells in uninjected mice or control mice injected with serum/saline or serum/saline containing 1 ng/mL of endotoxin. Each point represents the mean data from six fields randomly selected from an individual liver section.

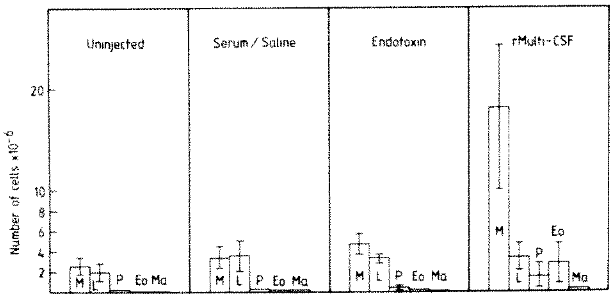

Fig 6. Total peritoneal cavity cells in C57BL mice injected for 6 days with recombinant multipotential colony-stimulating factor (rMulti-CSF) as compared with levels in uninjected mice or control mice injected with serum/saline or serum/saline containing 1 ng/mL of endotoxin; 12 mice per group. Mean values ± SD. M, macrophages; L, lymphocytes; P, polymorphs (neutrophils); Eo, eosinophils; Ma, mast cells.

philic and more vacuolated than were control peritoneal macrophages and occasionally were observed to have phagocytosed eosinophils. An increased level of mitotic activity was observed after 3 and 4 days of injection in peritoneal macrophages from mice injected with rMulti-CSF (mitoses per 10^3 cells: rMulti-CSF 7.4, serum/saline 0.9, uninjected 1.9); by day 7, however, the frequency of mitotic cells had fallen to control levels (mitoses per 10^3 cells: rMulti-CSF 1.2, serum/saline 1.2, uninjected <0.1).

Parallel studies using C3H/HeJ mice injected for 6 days also showed that rMulti-CSF induced major rises in macrophage, eosinophil, and neutrophil levels in the peritoneal cavity (Fig 7) although the absolute levels of these cells were lower than those observed in C57BL mice. Rises in peritoneal cell numbers in BALB/c mice were intermediate between those of C57BL and C3H/HeJ mice.

The injection of endotoxin (1 ng/mL) into mice of all three strains failed to elicit the large rises observed in mice injected with rMulti-CSF.

Changes in other organs. Histological sections of other organs from mice injected for 6 days with rMulti-CSF revealed no infiltration of mixed hematopoietic cells comparable with that observed in the liver. In the mesenteric lymph node, a 30-fold rise in C3H/HeJ mice and a 70-fold rise in BALB/c mice was observed in small, incompletely granulated mast cells in mice injected with rMulti-CSF as compared with control mice. No mast cell increase was observed in the mesenteric node of C57BL mice injected with rMulti-CSF. In the kidney, occasional small mast cells were observed in the outer cortex of mice injected with rMulti-CSF, but not in the cortexes of control mice. No mast cell infiltration was observed in the lung or the heart. In the small bowel, infiltration of additional small mast cells in the base and body of the villae was observed in C3H/HeJ and BALB/c mice injected with rMulti-CSF (threefold and 40-fold, respectively) but no infiltration was observed in C57BL mice. The thymus showed a small number of additional small mast cells in mice injected with rMulti-CSF.

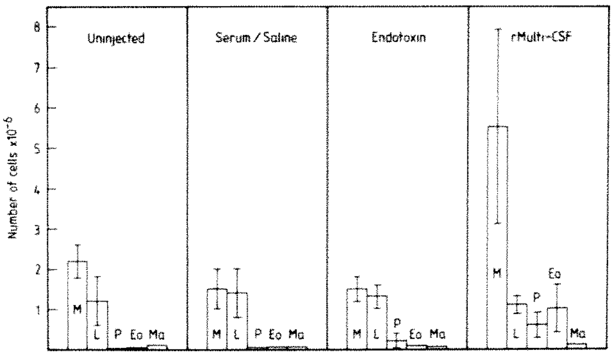

Fig 7. Total peritoneal cavity cells in C3H/HeJ mice injected for 6 days with recombinant multipotential colony-stimulating factor (rMulti-CSF) as compared with levels in uninjected mice or control mice injected with serum/saline or serum/saline containing 1 ng/mL of endotoxin; four mice per group. Mean values ± SD. M, macrophages; L, lymphocytes; P, polymorphs (neutrophils); Eo, eosinophils; Ma, mast cells.

In sections of flank skin, distant from the site of injections, a fivefold rise in small mast cells and a twofold increase in large mast cells was observed in C3H/HeJ and BALB/c mice injected for 6 days with rMulti-CSF as compared with control groups. Mast cells numbers were quite variable between individual mice within each group. No significant changes were observed in the skin content of mast cells in C57BL mice injected with rMulti-CSF.

Phagocytic activity of blood neutrophils and peritoneal macrophages. As shown in Table 8, a variable proportion of neutrophils from the peripheral blood of control mice exhibited phagocytic activity for antibody-coated erythrocytes, but cells from mice injected for 6 days with rMulti-CSF did not exhibit elevated phagocytic activity as assessed either by the percentage of phagocytic cells or the number of cells phagocytosed.

In contrast, macrophages from the peritoneal cavity of mice injected with rMulti-CSF exhibited a higher percentage of phagocytic cells and phagocytosed larger numbers of antibody-coated erythrocytes than did macrophages from control mice. Negligible phagocytosis was seen in the absence of opsonizing antibody. However, unlike the negative results with control injections using other parameters, the injection of serum/saline with or without added endotoxin increased the phagocytic activity of peritoneal macrophages. The effect of rMulti-CSF was distinguishable from that of endotoxin in the studies using endotoxin-hyporesponsive C3H/HeJ (Table 8). No differences were observed between the various groups in the phagocytic activity of peritoneal eosinophils, the percentage of phagocytic cells being low ($<10\%$) in all groups in which sufficient cells were available for scoring. No difference was observed between the phagocytic activity of peritoneal and blood neutrophils, and the injection of rMulti-CSF did not increase the activity of either population.

DISCUSSION

The IP injection of purified bacterially synthesized rMulti-CSF to normal adult mice produced detectable hematopoietic changes both at the site of injection and systemically. The nature of the responses observed and the cell types involved were in general agreement with expectations from

Table 8. Antibody-Dependent Phagocytosis by Peritoneal Macrophages and Blood Neutrophils From Mice Injected With rMulti-CSF

		C57BL		C57BL		C57BL		C3H/HeJ		BALB/c	
Cells	Strain/Treatment	Phag (%)	EA per 100 Cells	Phag (%)	EA per 100 Cells	Phag (%)	EA per 100 Cells	Phag (%)	EA per 100 Cells	Phag (%)	EA per 100 Cells
Peritoneal macrophages	Uninjected	6 ± 3	13 ± 8	4 ± 4	3 ± 1	6 ± 4	18 ± 5	33 ± 4	70 ± 4	34 ± 6	67 ± 18
	Serum/saline	19 ± 7	88 ± 27	14 ± 3	42 ± 13	34 ± 2	104 ± 8	65 ± 3	215 ± 10	58 ± 13	265 ± 39
	Endotoxin	NT	NT	NT	NT	45 ± 4	221 ± 29	54 ± 2	183 ± 9	56 ± 12	317 ± 34
	rMulti-CSF	44 ± 2	259 ± 11	39 ± 3	161 ± 6	44 ± 6	287 ± 67	73 ± 3	460 ± 39	67 ± 3	351 ± 35
Blood neutrophils	Uninjected	NT	NT	19 ± 1	22 ± 1	51 ± 3	72 ± 17	52 ± 3	71 ± 4	78 ± 4	124 ± 7
	Serum/saline	NT	NT	19 ± 2	22 ± 3	54 ± 23	95 ± 22	60 ± 4	78 ± 1	70 ± 3	105 ± 9
	Endotoxin	NT	NT	NT	NT	41 ± 7	78 ± 13	62 ± 4	89 ± 3	69 ± 3	118 ± 8
	Multi-CSF	NT	NT	19 ± 1	20 ± 2	44 ± 9	64 ± 1	67 ± 3	92 ± 7	60 ± 4	88 ± 5

In each experiment, pools of blood cells or peritoneal cells from four mice were used. Incubations to test for phagocytic activity were performed in triplicate or quadruplicate for each cell suspension. Data shown are percentages of phagocytic (Phag) cells as determined from counts on ≥600 cells and the total number of antibody-coated erythroid cells phagocytosed by 100 cells (EA/100 cells). In the third C57BL experiment, the erythrocytes were coupled with TNP and an anti-DNP antibody used. Groups are bracketed together where no statistically significant difference exists between the mean values by Tukey's method of least significant differences.

rMulti-CSF, recombinant multipotential colony-stimulating factor.

the actions of Multi-CSF as established from in vitro studies.[1-7]

Based on the assays of serum and peritoneal cavity fluid, a single injection of 102,000 U of rMulti-CSF only achieved significantly elevated (>50 U/mL) concentrations of Multi-CSF for 2½ hours in serum and 4 hours in the peritoneal cavity. With injections at intervals of 8 to 10 hours, this would have left the hematopoietic populations unstimulated for a major portion of each 24-hour period, and it is likely that the responses observed were below those that could have been achieved by using an indwelling osmotic pump or continuous intravenous infusion. However, it has been observed that occupancy of Multi-CSF receptors on bone marrow cells is rapid, essentially irreversible, and followed by a relatively slow reexpression of full membrane receptor numbers (N.A. Nicola, and L. Peterson, unpublished data, June 1985). Therefore it may be that the interrupted injection schedule used was less inefficient than might have been supposed.

In vitro, the effects of Multi-CSF on progenitor cell numbers are complex because of two opposing effects: (a) a decrease in progenitor cell numbers due to stimulation of these cells by Multi-CSF to form maturing progeny, and (b) an increased generation of progenitors by stimulation of stem cell cycling with progenitor cell formation.The net results of these opposing effects may well differ according to the Multi-CSF concentration used or to the cell population studied, eg, spleen *v* bone marrow. In the present in vivo experiments, no effects were observed on progenitor cell numbers or types in the marrow; in the spleen, however, twofold to tenfold rises were observed in granulocyte-macrophage, eosinophil, and erythroid progenitor cell levels with up to an 18-fold increase in CFU-E levels. In preliminary experiments using C57BL mice, the injection of rMulti-CSF for 6 days did not alter CFU-S levels in the marrow, but elevated the frequency of CFU-S fivefold in the spleen. These results are in general agreement with those of a similar study using bacterially synthesized Multi-CSF.[11] In can be concluded, therefore, that the regimen of injections used did not restrict the production of maturing cells by causing a depletion in progenitor cell numbers. On the contrary, it caused a significant rise in progenitor cells, at least in the spleen, an organ in the mouse noted for its ability to expand progenitor cell populations in situations involving increased hematopoiesis.[23-26]

Changes in maturing cell populations in the marrow and spleen reflected the above pattern. In the marrow, total cell numbers declined slightly, and no subset of cells showed a significant rise in frequency. In contrast, the spleen exhibited a consistent 50% weight increase and twofold to fourfold rises were observed in the percentages of immature granulocytes, eosinophils, nucleated RBCs, and megakaryocytes. The mast cell responses in the spleen to the injection of rMulti-CSF were quantitatively the largest, involving rises of tenfold to 100-fold in the population of small, incompletely granulated mast cells, these cells commonly developing in clusters resembling in vitro colonies. The mast cell response showed marked variation between the three mouse strains studied, being high in C3H/HeJ and BALB/c mice and much less prominent in C57BL mice, and were selective in that changes in the numbers of large mature mast cells were quantitatively much smaller (ranging from no change to a twofold to fourfold rise). The mast cell population changes in the spleen provided the most convincing single piece of evidence that injected rMulti-CSF was able to act systemically on hematopoietic target cells in normal hematopoietic organs.

The liver of the adult mouse normally contains no significant hematopoietic populations other than Kupffer cells, but the liver is an active site of hematopoiesis in fetal and neonatal life, and hematopoiesis can return to the liver in conditions of stress or damage to the bone marrow or spleen.[27] In this context, it is of interest that the liver was the only organ to display obvious infiltration by a mixed hematopoietic population in mice injected with rMulti-CSF. The infiltrating cells were at varying stages of maturation but exhibited no mitotic activity and presumably had seeded in the liver from the circulation.

The most striking overall population changes observed in mice injected IP with rMulti-CSF were in the peritoneal cavity itself. Here the population of macrophages rose sixfold, eosinophils rose 15-fold, and neutrophils rose sevenfold. Early in the injection period, increased mitotic activity was observable in the peritoneal macrophages from mice injected with rMulti-CSF; this declined, however as injections continued, either due to an inability of the cells to respond to further stimulation or, possibly, because migrating cells entering the cavity were nonresponsive. Mitotic activity in peritoneal macrophages could have accounted for some but probably not all of the rise in peritoneal macrophages and, since the increased neutrophils and eosinophils were exclusively postmitotic mature cells, it can be concluded that much of the increase in peritoneal cavity cellularity was owing to an influx of cells from the circulation. Human GM-CSF has been shown to inhibit neutrophil migration,[28] and a high local concentration of CSF might therefore encourage an accumulation of migratory cells. Because the spleen, bone marrow, and liver showed either no change or absolute rises in the populations of these cells, it is likely that the hematopoietic population rises in the peritoneal cavity and organs indicate a substantially increased production of mature cells during the 6-day injection period. Levels of circulating cells in the blood showed only moderate elevation, implying a more rapid transit of cells through the blood to seed in the peritoneal cavity and other tissues, an effect noted in early studies on mice injected with crude CSF-containing material.[29]

The macrophages from the peritoneal cavity of mice injected with rMulti-CSF exhibited increased phagocytic activity for antibody-coated erythrocytes and a morphology consistent with an activated state, eg, basophilia and vacuolation. Exposure of monocytes and macrophages in vitro to GM-CSF[30] or Multi-CSF[31] has been shown to increase their phagocytic activity, and a comparable stimulation by rMulti-CSF may have been the basis for the present in vivo effects. However, the effects could have been produced by some other biologically active mediator induced by rMulti-CSF or may even have been caused by the selective migration to the peritoneal cavity of a more active population of macrophages. Although exposure of neutrophils and eosinophils in vitro to some types of CSF induces increased functional activity,[32] no increased phagocytic activity by these cells was observed in the present experiments; it has yet to be established whether Multi-CSF can stimulate increased phagocytic activity by neutrophils and eosinophils in vitro.

In vivo studies of this type carry a known hazard that some or all of the changes observed may have been caused by contaminating endotoxin, the more so since the recombinant Multi-CSF used was synthesized by *E coli* and because endotoxin in high concentrations is capable of elevating progenitor cell levels in the spleen.[24,25] Throughout the present experiments, two types of control were used to exclude the possibility that the observed changes were due to endotoxin: (a) the use of a control group injected with at least 16-fold higher endotoxin concentrations than was detectable in the rMulti-CSF preparations, and (b) the use of endotoxin-hyporesponsive C3H/HeJ mice[33] to confirm all major observations. Several lines of evidence argue against the observed changes being due to endotoxin: (a) the recombinant Multi-CSF used was purified to homogeneity and contained no detectable endotoxin (<0.06 ng/mL); (b) injected endotoxin at higher concentrations (1 ng/mL) failed to produce the observed changes in the spleen, peritoneal or liver populations; and (c) similar responses to injected rMulti-CSF were observable in endotoxin-hyporesponsive C3H/HeJ mice.

Could the changes induced by the rMulti-CSF preparation have been caused by some other bacterial contaminant? There are several arguments against this possibility. First, amino acid sequence analysis of bacterially synthesized CSF purified by the present method shows only a single sequence with no evidence of contaminating polypeptides (R. Simpson, unpublished data, September 1985). Second, the crude commercial endotoxin preparation used was also of *E coli* origin and was more likely to contain such contaminants, yet failed to induce the changes produced by rMulti-CSF. Third, recombinant GM-CSF also synthesized by *E coli* and purified using the same general procedures produces a different response pattern when injected into adult mice, inducing no rises in immature mast cells and a fall in marrow progenitor cells (D. Metcalf, C.G. Begley, and G. R. Johnson, unpublished data, December 1985). Because the injected rMulti-CSF stimulated the same populations known to respond in vitro to purified native Multi-CSF, it seems reasonable to conclude that the observed in vivo effects were caused by the injected rMulti-CSF and not some hypothetical contaminant.

Although the actions of Multi-CSF in vitro have been established as direct effects on responding hematopoietic populations, there is no proof that the observed changes in vivo were caused by direct actions of the injected rMulti-CSF. However, injected ^{125}I-labeled rMulti-CSF has been shown to bind to marrow cells in vivo (N.A. Nicola, and D. Metcalf, unpublished data, July 1985), making it possible that the observed changes were direct effects of the injected rMulti-CSF. A formal possibility remains that the effects observed could have been initiated by rMulti-CSF but mediated by some other mechanism, eg, by the rMulti-CSF acting as an antigen or by the rMulti-CSF inducing the formation of some other biologically active molecule. However the presence of membrane receptors for Multi-CSF on the cell types responding in vivo (N.A. Nicola, and D. Metcalf, unpublished data, July 1985) makes it more likely that the action of injected rMulti-CSF was a direct one on responding cells.

Do the data establish Multi-CSF as a "normal regulator" in vivo? Multi-CSF is not detectable in normal serum[34,35] and, although activated normal T lymphocytes can synthesize high levels of Multi-CSF in vitro,[36,37] it remains unclear whether any Multi-CSF is synthesized in a normal spleen even though this organ presumably contains some activated T lymphocytes. From the marked rise observed in small mast cells in the spleen of mice injected with rMulti-CSF and their low numbers in the normal spleen, it seems doubtful whether significant concentrations of Multi-CSF are produced locally in a normal spleen. The present experiments suggest that Multi-CSF may not be an important regulator in steady-state conditions but might function as a highly effective regulator in states of T lymphocyte activation and consequent Multi-CSF synthesis.

The present studies using rMulti-CSF have provided a basis for the expectation that the CSFs will prove of value clinically in the stimulation of increased hematopoiesis when comparable human-active CSFs become available for testing. The marked strain differences observed in mice injected with rMulti-CSF predict that not all human subjects would respond in an identical manner to such injected CSF. Finally, the increased numbers and functional activation of cells at the site of injected CSFs raise the interesting possibility that the injection of CSF in sites of local infection or inflammation may be an effective method for enhancing host reponses to such local lesions.

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Platelet von Willebrand Factor: An Important Determinant of the Bleeding Time in Type I von Willebrand's Disease

By Harvey R. Gralnick, Margaret E. Rick, Laurie P. McKeown, Sybil B. Williams, Robert I. Parker, Pascal Maisonneuve, Christine Jenneau, and Yvette Sultan

We studied 17 patients with moderate to mild type I von Willebrand's disease (vWd) and correlated the bleeding time with the plasma von Willebrand factor antigen (vWf Ag), the plasma vWf activity (ristocetin cofactor), the platelet vWf Ag, and the platelet vWf activity. We found an excellent correlation between the bleeding time and the platelet vWf activity and, to a lesser extent, between the bleeding time and the platelet vWf Ag. The length of the bleeding time was inversely proportional to the level of the platelet vWf ($P < .001$) or, to a lesser extent, the platelet vWf Ag ($P < .05$). The plasma vWf Ag and activity did not correlate significantly with the bleeding time. These studies indicate that the platelet vWf is one of the important bleeding time factors in type I vWd and that the platelet vWf plays an important role in the early steps of hemostasis.

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THE CONGENITAL coagulation disorder, von Willebrand's disease (vWd), is characterized by a long bleeding time, low levels of factor VIII coagulant activity (VIII:C), von Willebrand factor antigen (vWf Ag), and ristocetin cofactor activity (vWf activity).[1,2] The most common form of vWd, ie, type I, is inherited in an autosomal manner and has a concordant reduction of all of the above mentioned activities.[2,3] However, the bleeding time is quite variable and often does not correlate well with the VIII: C, plasma vWf Ag and/or, in general, with the ristocetin cofactor activity (RCof).[1,4] This has led some investigators to suggest that a separate bleeding time factor exists that is deficient in vWd.[1,4] In an attempt to identify a factor that might be related to the bleeding time prolongation in vWd, we studied 21 patients with type I vWd (including four patients with severe disease and 17 patients with moderate to mild clinical symptoms) and correlated the bleeding time with the plasma and platelet vWf Ag and activity as measured by the RCof assay. In this study, we found that a prolonged bleeding time had an excellent correlation with a decreased level of platelet vWf activity and that a normal bleeding time correlated with normal platelet vWf activity. The plasma vWf Ag and vWf activity showed a poor correlation with the bleeding time values.

These studies indicate that an important determinant of bleeding time in type I vWd is the platelet vWf activity and suggest that it plays an important role in the initial phases of the formation of the hemostatic plug.

MATERIALS AND METHODS

Each patient gave informed consent for these studies. None of the patients were taking drugs known to affect factor VIII or vWf Ag or activity. Blood was obtained with a 19-gauge needle using a two-syringe technique. Plasma preparation and separation of platelets from whole blood were performed as previously described.[5,6] Platelets were isolated from whole blood anticoagulated with 10.9 mmol/L of sodium citrate, 1 mmol/L of EDTA by centrifugation on a discontinuous arabinogalactan (Stractan, St. Regis Co, Takoma, Wash) gradient (3 mL 20% of 5 mL 10% Stractan) as previously described.[5] In some experiments, 5 mmol/L of EDTA, 6 mmol/L *N*-ethylmaleimide, and 1 mmol/L of leupeptin were added to the anticoagulant used for the separation of plasma and platelets. Platelets were washed free of Stractan with 0.01 mol/L of Tris, 0.15 mol/L of NaCl, 0.003 mol/L of EDTA, and 0.5% serum bovine albumin (BSA), pH 6.8. After centrifugation at 1,500 g for 10 minutes, the platelet pellet was resuspended in 0.01 mol/L of Tris, 0.15 mol/L of NaCl, and 3% BSA, pH 7.35, incubated at 37 °C for 15 minutes, and then counted. The platelet count was adjusted to 10^6 platelets per microliter by the addition of resuspending buffer, and the platelets were lysed by the addition of 1/40 vol of 20% wt/wt Triton X-100. The platelets were then frozen at −70 °C; before analysis, they were thawed and spun at 10,000 g for 30 minutes at 4 °C. The supernatant was removed and used in the assays for vWf Ag and vWf activity.

Plasma VIII:C activity, plasma and platelet vWf Ag, and vWf activity were measured as previously described.[7] One unit of activity is defined as that found in 1 mL of pooled normal plasma. The bleeding time was performed on the patients' forearms using a standardized template method. Bleeding times and plasma and platelet vWf studies were performed on at least two separate occasions.

The multimeric structure of the plasma and platelet vWf were studied by glyoxal agarose gel electrophoresis modified from the technique originally described by Hoyer and Shainoff.[8] The major modifications were a gel thickness of 0.5 mm and a final sample size of 10 μL. Equal volumes of normal and patient plasma and platelet lysate were electrophoresed on the gel (regardless of the antigen content).

Three of the four patients with severe type I vWd had no family history of a bleeding disorder, whereas one patient had one family member (brother) who died of hemorrhage without a diagnosis ever being established. The other 17 patients with type I vWd had a personal history of bleeding and had at least one immediate family member with a similar bleeding history.

Classical correlation coefficients were determined on a Model 10 Hewlett-Packard programmable computer.

RESULTS

The four patients with severe homozygous or double heterozygous vWd were shown to have plasma and platelet vWf Ag and vWf activity of <0.03 U/mL (Figs 1 and 2) and bleeding times >30 minutes. In contrast, the 17 patients with moderate to mild type I vWd had bleeding times that ranged from 4 to 27 minutes and VIII:C levels that varied between 0.08 TO 0.75 U/mL. The plasma vWf Ag ranged between

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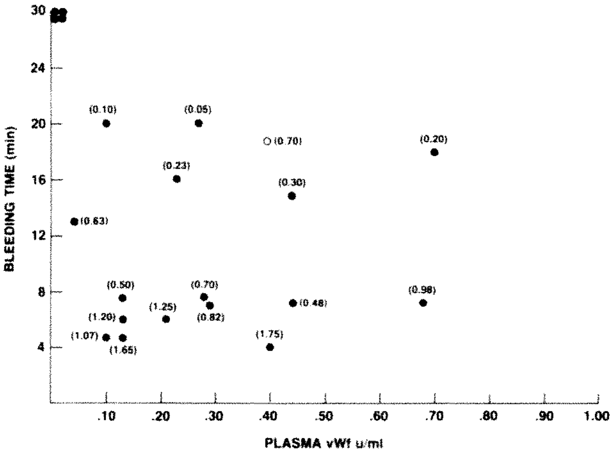

Fig 1. Comparison of the bleeding time with plasma von Willebrand factor (vWf) activity. The values in parentheses next to each of the closed circles represent the platelet vWf activity for that patient. The correlation of the plasma vWf activity and the bleeding time was very poor in that several patients had normal plasma vWf activity with long bleeding times and several patients had very low plasma vWf activity with normal bleeding times. The upper limit of normal for the bleeding time was 8 minutes; the lower limit of normal for the plasma vWf activity was 0.48 U/mL.

0.11 to 0.70 U/mL, and the plasma vWf activity varied between 0.05 to 0.70 U/mL (Table 1). Sixteen of the 17 patients had plasma vWf Ag below the normal range, and 15 patients had decreased plasma vWf activity. The platelet vWf Ag value varied between 0.22 to 1.30 U/10^9 platelets and the vWf activity varied between 0.05 to 1.75 U/10^9 platelets. Only 2 of the 17 patients had a decrease in platelet vWf Ag, whereas 5 patients had a decrease in their platelet vWf activity (Table 1).

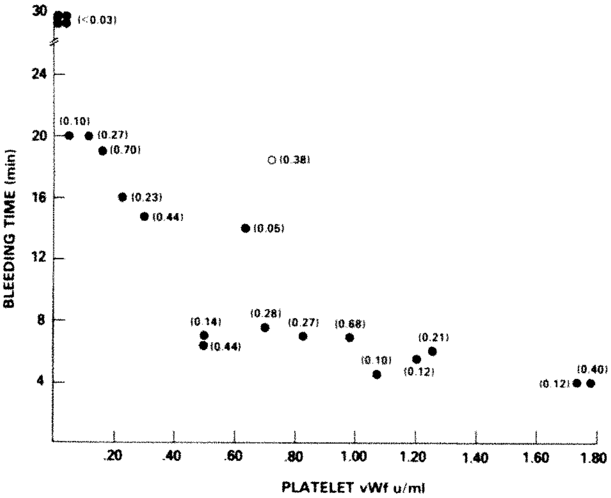

Fig 2. Correlation of the bleeding time and platelet von Willebrand factor (vWf) activity. All patients with moderate type I vWd who had low platelet vWf activity had prolonged bleeding times. Only two with normal platelet vWf activity had prolonged bleeding times. One of these patients (O) has an associated platelet aggregation defect; in the other plasma, vWf activity was 0.5 U/mL. The upper limit of normal for the bleeding time was 8 minutes; the lower limit of normal for the plasma vWf activity was 0.42 U/mL.

The correlation coefficients determined for the 17 patients with moderate to mild vWd are as follows. There was no correlation between the bleeding time and the VIII:C (data not shown), nor was there a correlation between the bleeding time and the plasma vWf Ag ($r = .2034$, $P > .10$) or vWf activity ($r = .1677$, $P > .10$) (Fig 1 and Table 1). In contrast, the relationship between the platelet vWf activity and the bleeding time revealed a remarkably good correlation ($r = .8055$, $P < .001$), whereas the correlation of the bleeding time with the platelet vWf Ag was significant but less than that observed with the platelet vWf activity ($r = .4895$, $P < 0.05$). The results of the duplicate analyses were within 1 to 2 minutes for the bleeding time and 10% to 16% for the plasma and platelet vWf studies.

The five patients who had decreased amounts of platelet vWf activity all had prolonged bleeding times, whereas 10 of the 12 patients with normal platelet vWf activity had normal bleeding times (Fig 2). The two patients who had normal levels of platelet vWf activity and prolonged bleeding times were somewhat unique. One of these patients (patient 4, Table 1) had a bleeding time of 17 minutes despite having a platelet vWf of 0.70 U/10^9 platelets. When he was given 1-deamino-8-D-arginine vasopressin (DDAVP), his bleeding time shortened to 10 minutes with a rise in his plasma VIII:C, vWf Ag, and RCof activity to normal levels. Later, he was restudied and was shown to have a platelet storage pool defect. The second patient had normal levels of vWf Ag and vWf activity in her platelets but had the most severe deficiency of plasma vWf (0.05 U/mL) and factor VIII:C. This woman had a bleeding time of 12.5 minutes, much shorter than would be expected from her plasma vWf levels. She had undergone five major surgical procedures (including a tonsillectomy, hysterectomy, and two spontaneous deliveries) with no excessive bleeding. Of the 17 patients with type I vWd listed in Table 1, patients 14, 15, and 16 are the father and two sons in one family, respectively; and patients 6 and 11 are the mother and son from another family.

Glyoxal agarose electrophoresis of the plasma and platelet vWf in the 17 type I patients revealed a multimeric pattern qualitatively indistinguishable from normal. There appeared to be a marked decrease in the plasma vWf Ag content in almost all patients and in the platelet vWf content of five patients (Fig 3). The presence of protease inhibitors in the anticoagulant did not modify the multimeric organization, vWf Ag levels, or vWf activity of the plasma or platelets.

DISCUSSION

In the first description of vWd, Willebrand noted that all of the affected family members had prolonged bleeding times; this became a prerequisite for the diagnosis of the disease which bears his name.[9] A 1968 study of 37 patients with vWd revealed that two patients had normal bleeding times, and a large study in 1977 revealed that 15 of 83 vWd patients with disease of moderate to clinical severity had normal bleeding times.[4,10] One study has suggested a good correlation between plasma vWf activity and the bleeding time,[11] whereas other investigators have found a poor correlation between the bleeding time and the plasma vWf Ag or

Table 1. Type I von Willebrand's Disease, Dominant Inheritance

			Plasma		Platelet	
Patient No.	Bleeding Time (min)	F.VIII (U/mL)	vWf Antigen U/mL	vWf Activity U/mL	vWf Antigen U/10⁹*	vWf Activity U/10⁹*
1	27	0.09	0.12	0.10	0.22	0.10
2	20	0.40	0.23	0.27	0.25	0.05
3	18	0.47	0.70	0.70	0.46	0.20
4	17	0.54	0.32	0.38	0.46	0.68
5	16	0.30	0.32	0.23	0.13	0.23
6	15	0.31	0.46	0.44	0.35	0.30
7	12.5	0.08	0.11	0.05	0.37	0.63
8	7.5	0.59	0.45	0.28	0.36	0.70
9	7	0.47	0.27	0.29	0.32	0.82
10	7	0.29	0.48	0.68	1.30	0.98
11	6	0.16	0.14	0.13	0.25	0.50
12	6	0.41	0.46	0.21	0.60	1.25
13	6	0.62	0.32	0.44	0.28	0.47
14	5	0.25	0.14	0.12	0.81	1.20
15	4	0.17	0.13	0.10	0.65	1.07
16	4	0.13	0.14	0.12	1.25	1.65
17	4	0.75	0.28	0.40	0.46	1.75
Normal	<8					
Mean ± 1 SD†			1.01 ± 0.18	1.07 ± 0.30	0.34 ± 0.14	0.77 ± 0.25
Observed range		0.52–1.58	0.55–2.13	0.75–1.50	0.15–0.58	0.41–1.28

*Units per 10^9 platelets.

†Studies on 20 normal subjects: 10 male, 10 female.

activity.[4,12] The inability to find a constant correlation between laboratory parameters and the bleeding time led to speculation concerning the factor(s) involved in the bleeding time in vWd.[1,4,12,13]

We studied 17 patients with heterozygous type I vWd and found an excellent correlation between the level of the platelet vWf activity and the bleeding time; the correlation between the platelet vWf Ag and the bleeding time was significant but not as strong as that seen with the platelet vWf activity. In contrast, the plasma levels of vWf Ag and/or activity did not correlate with the bleeding time. These studies indicate that the platelet vWf is a major factor in determining the bleeding time in the nontreated state in type I vWd. Our studies do not exclude an additive or independent role for plasma vWf but indicate that it appears to play a less significant role than that of the platelet vWf.

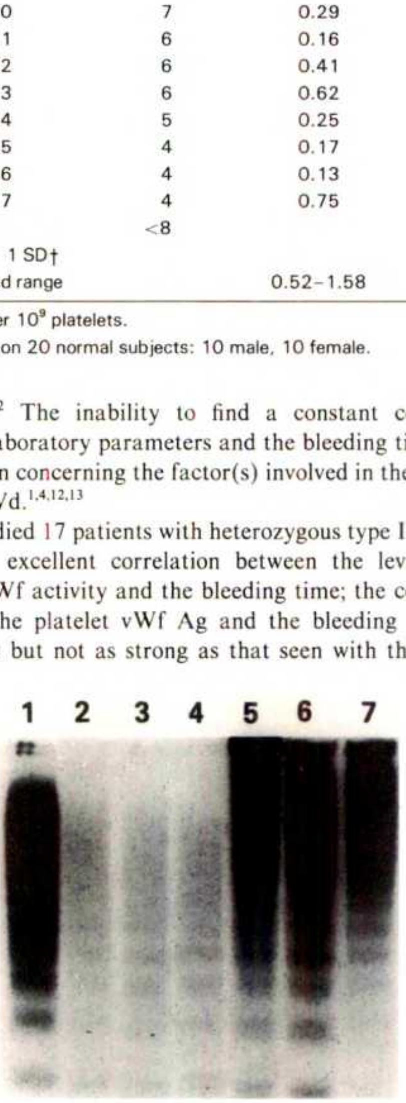

Fig 3. Agarose gel electrophoresis of the plasma and platelet vWf. Lane 1 is normal plasma; lanes 2, 3 and 4 are the plasma from a patient (patient 13, Table 1) collected in sodium citrate anticoagulant, in the same citrate anticoagulant with EDTA, and in sodium citrate, EDTA, and protease inhibitors, respectively. Lanes 5 and 6 are the patients' platelet vWf; blood in lane 5 was collected in sodium citrate and EDTA; in lane 6, it was collected in sodium citrate, EDTA, and protease inhibitors. Lane 7 shows normal platelet vWf for comparison.

Other studies reported subsets of patients with type I vWd who had normal platelet vWf Ag and in some cases vWf activity,[14,15] but no correlation was made between these values and any clinical parameter until recently. Mannucci and co-workers recently described six patients with type I vWd who had normal platelet vWf Ag and vWf activity. Two of these patients had normal resting bleeding times, and all six patients had a normal bleeding time after DDAVP infusions.[16] The patients with type I platelet low or platelet discordant vWd had prolonged bleeding times, and although the bleeding time shortened after DDAVP infusion, it did not become normal.[16] Ratnoff and Bennett reported that the bleeding time remained prolonged in three vWd patients in whom the VIII:C, vWf Ag, and plasma vWf activity rose to normal. It seems likely that in those patients the platelet vWf was still diminished and not affected by the pregnant state, by plasma product transfusion, or by the stress of hemorrhage.[12]

Our studies suggest that the platelet vWf is an important determinant in the bleeding time and that either the platelet membrane vWf or the vWf which is released after platelet stimulation acts as the initial bridge between platelets and the vessel wall, causing adhesion of these platelets to the vessel, or facilitates platelet–platelet interaction after adherence to the vessel wall.[17]

The findings of decreased levels of plasma vWf Ag and activity and normal or increased platelet vWf Ag and activity in some type I patients suggests that the genetic defect(s) that causes a decrease in the plasma vWf protein and its activities does not necessarily affect the platelet vWf and its activities. Further studies of type I vWd patients should include measurement of platelet vWf Ag and vWf activity. These studies focus on the importance of the platelet vWf activity in the determination of the bleeding time in vWd during a steady state. Further studies are necessary to identify the source and quantity of the vWf bound to subendothelium and the importance of the endothelial cell vWf and other cytoadhesive proteins in the determination of the bleeding time.

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An Antigen Common to Chronic Lymphocytic and Hairy Cell Leukemia Cells Not Shared by Normal Lymphocytes or by Other Leukemic Cells

By Julia F. Agee, Fred A. Garver, and Guy B. Faguet

Rabbit xenoantisera and mouse monoclonal antibodies prepared against human chronic lymphocytic leukemia (CLL) B cells were found to react against a single polypeptide chain with a mol wt of 69 kd found on leukemic cells of all CLL (N = 40) and B type hairy cell leukemia (HCL) patients (N = 9) examined. This common CLL-associated antigen (cCLLa) was not detectable on circulating T or B lymphocytes, thymocytes, lymph node and splenic lymphocytes, or bone marrow leukocytes from normal persons. In addition, the cCLLa was not detectable on cultured T or B lymphoblastoid cell lines or on malignant cells from other forms of lymphocytic or myelocytic leukemia. Non-Hodgkin's lymphoma cells also failed to express the antigen. Autologous cultured lymphoblastoid cell lines were established from residual normal B cells from a CLL patient whose cells were used to generate one of the antisera. Absorption of the antibody with these cultured polyclonal B cells did not affect the anti-CLL activity, which suggests that the cCLLa is not HLA related. Unlike the T cell differentiation complex gp65-71, the cCLLa was not expressed on fetal or cord blood lymphocytes or on mitogen-stimulated normal lymphocytes and was distinct from the antigen recognized by the LEU-1 antibody in spite of their similar mol wt. The cCLLa was also determined to be unrelated to the human T cell leukemia lymphoma virus (HTLV-1). One of the monoclonal antibodies generated against the cCLLa was a complement binding IgG which exhibited highly selective cytotoxic activity against 100% of cells bearing the cCLLa. Such an antibody might prove clinically useful in early diagnosis and treatment of CLL and HCL.

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DEFINING the expression of normal antigens on the surface of neoplastic hematopoietic cells has facilitated the elucidation of the cellular origin and differentiation pathways of these cells. Thus, leukemias and lymphomas, traditionally grouped according to histology, cell morphology, and cytochemistry, can now be subclassified based on their membrane markers.[1,2] Cells from patients with acute lymphoblastic leukemia (ALL), for example, exhibit T or B lymphocyte markers or lack both, the latter being associated with a better prognosis.[2-3] In contrast, malignant cells of most patients with chronic lymphocytic leukemia (CLL) express phenotypic markers characteristic of B lymphocytes.[4] The SIg of CLL cells with B phenotype has restricted heterogeneity as compared with that found on normal B cells, indicating a monoclonal origin of the leukemic cell.[5] Although the evidence supporting the existence of distinct tumor specific antigens in humans is circumstantial, efforts were made to identify surface antigens uniquely expressed on leukemic cells.[6-8] However, early reports of tumor-associated antigens on leukemic cells were met with skepticism in view of their lack of specificity. This and the observation that leukemia appears to be a consequence of a blocked differentiation rather than a loss of growth control[9,10] suggest that tumor-associated antigens are probably remnant "differentiation" antigens deleted during the differentiation process of normal cells.

In this communication, we report that CLL and hairy cell leukemia (HCL) share a common but unique antigen which appears distinct from differentiation antigens and is unrelated to antigens previously detected on leukemia or lymphoma cells, including the HTLV-1.

MATERIALS AND METHODS

Cells. Normal and CLL lymphocytes and blasts from leukemic patients were prepared from heparinized peripheral blood by Ficoll-Hypaque gradient modified to remove monocytes.[11] CLL cells were obtained from patients with B cell lymphoproliferations with circulating lymphocyte counts $>30{,}000/\mu L$. Promyelocytes were obtained from the buffy coat fraction after dextran sedimentation of venous blood from patients with acute promyelocytic leukemia (APrML). Thymic, splenic, lymph node, and bone marrow cells were teased from fresh biopsy specimens. Erythrocytes were removed from bone marrow samples by hypotonic lysis. B-enriched and T-enriched lymphocyte populations were separated according to the method of Weiner and colleagues.[12] B-enriched cells were in some instances prepared by the HEAC rosetting technique.[13] B-enriched and T-enriched lymphocyte preparations were consistently >80% and >90% phenotypically homogeneous, respectively. Myeloid and monocytoid cell contamination averaged <3%, respectively, as illustrated in Table 1. Lymphoid cell lines were established from peripheral blood lymphocytes of a CLL patient and of two patients with HCL as outlined by Moore.[14] Lymphoblastoid cell lines of B-cell (LS-1, MA-1, SA-1, KS-1, DBA-2, DBA-3, JY, B66, B110, B411, RPMI-1788, IM9, SB, and QIK) or T cell lineage (HSB, CEM, TAR, and MOLT-4), the pluripotent K562 cell line, and a fetal lymphoid cell line were cultured in appropriate media and stored in liquid nitrogen. Target cell preparations were characterized by cytomorphology and by surface marker phenotyping prior to assaying. Cell subset distribution in cell preparations is indicated in the text when appropriate.

Cell phenotyping. SIgs were determined by direct immunofluorescence[6] using fluorescein-conjugated antisera specific for IgM, IgG, IgA, and IgD heavy chains and for κ and λ light chains (Meloy Laboratories, Springfield, Va). Other surface determinants were assessed by indirect immunofluorescence assay (IFA) using appropriate monoclonal antibodies commercially available: (OKT series, Ortho Diagnostics, Raritan, NJ; LEU series, Becton Dickinson, Mountain View, Calif; and My$_4$, B$_2$, MO$_2$ and J-5, Coulter Immunology, Hialeah, Fla).

Anti-CLL antibodies. Xenogeneic antisera were produced in New Zealand white rabbits by biweekly intravenous (IV) injections

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Table 1. Reactivity of CLL Cells and of Enriched Normal T and B Lymphocytes Before and After Incubation with CLL

				Preincubated†			Preincubated†		
	Fresh*			Goat Anti-Mouse			Goat Anti-Rabbit		
Phenotype	CLL	T	B	CLL	T	B	CLL	T	B
T Cells									
OKT-3	8.3	93.4	6.2	8.1	93.0	6.2	85.6	0.4	3.9
OKT-4	4.7	60.8	4.3	—	—	—	—	—	—
OKT-8	5.2	32.9	2.8	—	—	—	—	—	—
B Cells									
SIg	88.5	3.0	87.0	—	—	—	—	—	—
LEU-12	87.0	3.0	89.0	—	—	—	—	—	—
Monocytes									
Mo2	0.5	1.0	3.0	—	—	—	—	—	—
My4	1.0	1.0	2.0	—	—	—	—	—	—
Myeloid									
LEU-M4	1.0	1.0	1.0	—	—	—	—	—	—
Miscellaneous									
J-5	1.5	0.5	2.0	0.0	0.9	2.4	84.4	0.5	1.4
HTLV-1‡	1.0	1.4	3.0	0.0	0.5	1.0	88.4	0.9	2.5
HTLV-1§	1.5	1.0	2.5	2.0	1.0	0.9	85.1	1.5	1.9
LEU-1	88.3	92.0	3.3	83.9	95.3	1.4	87.3	1.8	1.0
CLL-1	85.1	1.4	3.3	2.9	1.4	2.9	86.3	1.0	1.4
αCLL	82.5	0.5	2.3	1.7	0.5	2.3	85.6	0.5	1.8

CLL, chronic lymphocytic leukemia; HTLV-1, human T cell leukemia lymphoma virus.

*Fresh cells were phenotyped by indirect immunofluorescence assay (IFA) using FITC-conjugated goat anti-mouse monoclonal antibodies (except for αCLL, which uses FITC-conjugated goat anti-rabbit antibody). SIg was assayed with FITC-conjugated goat anti-human antibody in a direct IFA.

†Cells were preincubated with αCLL prior to assaying in an indirect IFA using FITC-conjugated goat anti-mouse or goat anti-rabbit antibodies.

‡,§Mouse monoclonal antibodies against the HTLV-1 viral core proteins p19 and p24, respectively.

of 1 × 10^8 viable lymphocytes each from a single patient with B cell type CLL for 10 weeks with a final booster injection 2 weeks later. The antisera were heat-inactivated: 10 μL Trasylol (Aprotenin, Mobay Chemical Corp, NY) protease inhibitor was added, and anti-ABO antibodies were removed by absorption with 5 vol of AB erythrocytes. The sera were then individually absorbed with peripheral blood lymphocytes (PBL) (2.0 × 10^8/mL of antiserum) from each of three normal individuals who shared two or more HLA antigens with the antisera-generating CLL donor. The combined absorptions accounted for all of the HLA A, B, and C antigens detected on cells from each CLL donor. One absorbed antiserum (αCLL/αB) no longer reacted with T-enriched lymphocytes (92% E rosette-positive cells) but still reacted with CLL cells and with B-enriched lymphocytes (85% SIg-positive cells) from some normal donors. The latter reactivity was removed by absorption with either 3.0 × 10^8 B cells (87% SIg-positive cells) obtained by leukopheresis (cell centrifuge model 2997, IBM, Endicott, NY) of a normal subject whose B cells were reactive with antiserum αCLL/αB, or with 1.5 × 10^9 B cells from a lymphoblastoid culture line likewise reactive with antiserum αCLL/αB. After dialysis against 0.9% NaCl, the B cell-absorbed antiserum (αCLL) was centrifuged for 1 hour at 100,000 g to remove aggregated immunoglobulin. Alternatively, absorption of antiserum αCLL/αB with 0.5 × 10^9 CLL cells per milliliter from certain CLL patients generated an antiserum (αB) reactive against ~40% of B cells from 50% of normal individuals. Monoclonal mouse anti-human antibodies were also generated against CLL according to a modification (Faguet and Agee, manuscript in preparation) of the method of Kohler and Milstein.[15] Data of interest derived from one such antibody (CLL-1) are included in this report. All antibody preparations were used at titers sufficient to provide saturated binding conditions for all cells assayed. This was confirmed as follows: a first aliquot of CLL cells was incubated with αCLL or CLL-1 and a second aliquot of fresh CLL cells was exposed to supernatants of the first incubations. Both sets of cells assayed by IFA showed reactivity of comparable magnitude.

Immunofluorescence and cytotoxicity assays. Antibody reactivity against cells in suspension was determined by IFA[16] using fluorescein-labeled goat anti-rabbit IgG (Meloy) and goat anti-mouse IgG (Capel Laboratories, Cochranville, Pa) and by antibody-mediated cell lysis measured by ^{51}Cr-release cytotoxicity.[17] Antibodies were used in concentrations sufficient to achieve saturation binding. In each instance, nonimmune rabbit serum or irrelevant Ig were used as negative controls and to assess nonspecific binding and cytotoxicity. Appropriate commercially available monoclonal anti-T and anti-B cell antibodies were used as positive controls for both IFA and cytotoxicity studies of lymphoid target cells. Negative controls gave reactivity ranges of ≤ 4% and ≤ 10% for IFA and cytotoxicity, respectively. Positive controls gave reactivity ranges of 87% to 99% and 91% to 99% for normal B and T lymphocytes, respectively, and 64% to 100% for CLL cells regardless of assay used. Specific antimyeloid and antimonocytoid monoclonal antibodies were used mostly to ascertain the extent of nonlymphoid cell contamination of normal or CLL lymphocyte preparations.

Membrane preparation and partial purification. Membranes were prepared from leukemic cells obtained by leukopheresis of CLL patients with a WBC count of ≥ 300,000/μL according to a modified[18] published procedure.[19] Lyophilized samples from αCLL-IgG-Sepharose affinity column-purified membranes were dissolved in 10 nmol/L of phosphate buffer, pH 8.0, and applied to a 10-mL column (0.72 × 25 cm) of diethylaminoethanol (DEAE)-cellulose (DE-52, Whatman, Inc, Pierce Chemical Co, Rockford, Ill) equilibrated with the same buffer. The protein was eluted with a NaCl gradient made from 20 mL of .02 mol/L of NaCl and 20 mL of 2 mol/L of NaCl, followed by 10 mL of 3 mol/L of NaCl. One-milliliter fractions were collected, and the protein was located by measuring the absorption at 280 nm.

Gel electrophoresis. The homogeneity and mol wt of the antigen was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% slab gels.[20] The antigen was dissolved in .0625 mol/L of Tris-HCL, pH 6.8, containing 0.005% bromophenol blue, 2% SDS, 2% mercaptoethanol, and 10% glycerol, and heated at 100°C for 3 to 5 minutes. Mercaptoethanol and heating treatment were omitted for nonreduced samples. The gels were fixed and stained with Coomassie blue or silver nitrate. The mol wt of protein bands was estimated by extrapolation from a standard curve (mol wt v mobility) using mol wt markers run simultaneously.

RESULTS

Antibody reactivity of antiserum αCLL/αB. Antiserum αCLL/αB was titered against CLL and normal B lymphocyte target cells as shown in Fig 1. Increasing the antiserum concentration-target cell ratio resulted in greater lysis of CLL cells than of B cells. At the maximum amount of antiserum added, 50 μL, lysis of B cells reached a plateau of 60%, whereas 20 μL of antiserum achieved 100% lysis of malignant CLL cells (cells bearing monoclonal SIg). These results suggested that the antiserum contained different antibodies reacting with both CLL cells and normal B cells,

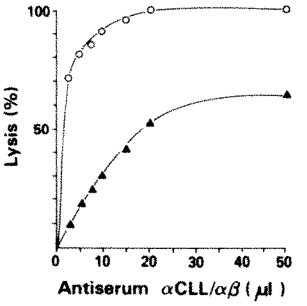

Fig 1. Titration of antiserum αCLL/αB by microcytoxicity assay with ^{51}Cr-labeled chronic lymphocytic leukemia (CLL) (o) or normal B (▲) target cells (5 × 10^4 per well). Percentage of lysis is expressed as a function of antiserum volume per well.

or that CLL cells exhibited either greater density of an antigen shared by normal B cells or greater affinity for a common antibody or were more susceptible to complement-mediated lysis than normal B cells were. In an effort to distinguish among the above possibilities, aliquots of antisera αCLL/αB were absorbed with B cells from seven normal subjects or with CLL cells from six patients and were assayed by cytotoxicity against ^{51}Cr-labeled normal B and CLL target cells (Table 2). As shown, enriched B cells (81% SIg positive) from certain normal subjects (Roc, Ta, Gre, Dor) reacted with the unabsorbed antiserum whereas others did not (Ger, Me). Absorption with B cells from any one reacting subject eliminated the antibody activity against B cells from that person and all previously reacting persons. Absorption with B cells from previously nonreacting persons (Ger, Me) failed to remove antibody activity to previously reacting B cells. However, both antisera remained markedly cytotoxic to CLL target cells regardless of whether they had been absorbed with reacting or nonreacting B cells. In contrast, absorption with CLL cells from some patients (Mil, Tay), abolished the antibody reactivity against both previously reacting normal B and CLL cells. Cells from other CLL patients (Bro, Wo, Lo, Tho) did not absorb out the antibody activity against previously reacting B cells, but did remove all antibody reactivity against CLL cells (eg, Lo). Furthermore, absorption of the antiserum with five times (2.5 × 10^8/0.1 mL) the number of normal B cells required to remove B cell antibody activity failed to remove cytotoxicity to CLL cells. These observations suggest distinct antigens on CLL and normal B cells rather than greater density on CLL cells of an antigen common to both. Thus, residual reactivity with Tay CLL target cells after absorption of antiserum αCLL/αB with CLL cells from Bro, Wo, Lo, and Tho is attributable to an antibody against the normal B cell antigen, which is absent on the absorbing CLL cells. Further studies conducted on B-enriched cells from normal donors (N = 25) and CLL cells from CLL patients (N = 26) indicate that this antibody identified a B cell subset (40%) found in ~50% of both normal subjects and CLL patients. Cell cultures were derived from peripheral blood of the CLL patient whose leukemic cells were originally used to generate antiserum αCLL/αB. Cells from one of these cultures expressed diverse SIgs (58% μ, 36% γ, 6% α, 63% κ and 31% λ), suggesting their polyclonal β cell origin. Although 82% of these cells reacted with the αB antiserum, only background reactivity (<4%) was seen with the αCLL antiserum (Table 3). As expected, absorption of the αCLL and αB antisera with these cells (4 × 10^7 cells/0.1 mL) did not affect the cytotoxicity of the αCLL for CLL target cells but abolished the lytic

Table 2. Cytotoxic Activity of Antiserum αCLL/αB After Absorption With Enriched Normal B Lymphocytes or CLL Cells

	Target Cells								
	Normal B Lymphocytes						CLL Cells		
Absorbing Cells	ROC	TA	GRE	DOR	GER	ME	TAY	LO	THO
None	27	25	21	22	5	4	94	85	96
Normal B Cells									
ROC	0	0	4	—	—	—	—	78	94
TA	4	0	2	—	—	—	—	66	92
GRE	5	8	8	—	—	—	—	65	100
DOR	—	—	2	—	—	—	—	—	94
GRI	4	0	0	—	—	—	—	81	100
GER	26	25	19	—	—	—	—	81	100
ME	27	24	24	—	—	—	—	66	93
CLL Cells									
MIL	3	5	4	3	0	0	0	0	—
TAY	0	6	—	4	7	3	0	0	—
BRO	25	24	29	20	0	0	21	0	—
WO	25	26	28	22	4	2	24	0	—
LO	24	26	—	—	—	—	22	0	—
THO	25	25	22	21	0	0	25	0	—

CLL, chronic lymphocytic leukemia.

Normal B-enriched or CLL cells (0.5 × 10^8) were incubated with antiserum αCLL/αB (60 minutes at 15 °C) and centrifuged; cell-bound antibody was ascertained by ^{51}Cr-release antibody-dependent cytotoxicity asasy. Results are shown as percentage of lysis of triplicate determinations (SEM <2%). Values <10% are considered negative.

Table 3. Reactivity of the αCLL and αB Antisera After Absorption With Autologous Normal B Lymphocytes

	Unabsorbed		Absorbed	
Target Cells	αCLL	αB	αCLL	αB
Autologous B Lymphocytes				
AG	0	29	0	0
TA	0	72	0	0
BR	0	56	0	0
HAM	0	27	0	0
MIL	0	37	0	0
CLL Cells				
WA	74	0	71	0
HU	100	7	96	1
BU	77	0	75	0
BR	95	0	92	0
BUR	94	2	93	0
OCO	93	81	87	0
JAV	97	34	95	0
SEN	91	43	93	0
DU	96	44	91	0
WAT	100	50	92	2

After absorption with autologous B lymphocytes (αCLL and αB antisera were used in the ^{51}Cr-release assay against both enriched B or CLL target cells. Data are expressed as percentage of lysis of triplicate determinations (SEM <2%).

activity of the αB serum against both normal B cells and CLL cells initially reactive with the αB antiserum.

Specificity of the αCLL antibody. The specificity of the antibody preparation αCLL was further studied on a large panel of donors by both microcytotoxicity and immunofluorescence assays. As illustrated in Table 4, the αCLL antibody reacted with all 40 B-CLL patients tested with a range of lysis of 64% to 100% and a range of fluorescence of 73% to 100%. Included in this group were three patients with prolymphocytic leukemia (PLL). SeD, a cell line derived from a patient with CLL[21] also showed reactivity with the αCLL antibody. In addition, cells from nine patients with SIg-positive HCL were positive for the cCLLa, as were cell lines EH and HK derived from two HCL patients (Faguet and Agee, manuscript in preparation). To ascertain the identity of the antigenic determinant detected by the αCLL on CLL and HCL cells, the following studies were done: Saturating concentrations of αCLL were incubated with 10^6 B-CLL cells (93% monoclonal SIg positive) before and after absorption for 60 minutes at 15 °C with CLL or HCL cells or with T-enriched (96% OKT$_3$ positive) and B-enriched (83% SIg positive) lymphocytes from a normal donor at concentrations of 1.5×10^9/mL and assayed by IFA. CLL cell reactivity with unabsorbed antibody (92%) was completely abrogated by absorption with CLL (2%) or HCL cells (2%), but was unaffected by absorption with either normal T (93%) or B (92%) lymphocytes. Similar experiments using cells from two patients with HCL as target cells yielded comparable results. Neither CLL nor HCL cells with non-B phenotypes were available for testing.

Table 4. Reactivity of the αCLL Antiserum

Target Cells	Tests (N) Total	Positive	Reactive Cells*
Cells bearing cCLLa			
CLL (B phenotypes)	40	40	92(7)
CLL cell line (SeD)	1	1	35(4)
HCL (B phenotypes)	9	9	46(5)
HCL cell lines (EH and HK)	2	2	63(5)
Cells not bearing cCLLa			
Normal lymphoid cells (N = 77)			
PBL	25	0	0–4
T cells (97% [SEM 4] OKT3+)	10	0	0–4
B cells (85% [SEM 4] SIg+)	10	0	0–4
Thymocytes	2	0	0–4
Splenocytes	2	0	0–4
Lymph node cells	2	0	0–4
Bone marrow interface cells	4	0	0–4
Cord blood lymphocytes	10	0	0–4
PWM-stimulated PBL	4	0	0–4
PHA-stimulated PBL	4	0	0–4
CON-A-stimulated PBL	4	0	0–4
Malignant cells (N = 76)			
Leukemias	32	0	0–4
Acute leukemias	27	0	0–4
ALL (childhood)	5	0	0–4
ALL (adulthood)	9	0	0–4
AML	7	0	0–4
AMMoL	4	0	0–4
APrML	2	0	0–4
CML in blastic crisis	5	0	0–4
Lymphomas	24	0	0–4
Non-Hodgkin's lymphoma	19	0	0–4
PBL	14	0	0–4
Lymph node cells	6	0	0–4
Hodgkin's disease	5	0	0–4
Lymph node cells	3	0	0–4
Splenocytes	2	0	0–4
Cell lines	20	0	0–4
Fetal lymphoblastoid cell line	1	0	0–4
B lymphoblastoid cell lines	14	0	0–4
T lymphoblastoid cell lines	4	0	0–4
Pluripotent cell line (K-562)	1	0	0–4

Reactivity of the αCLL against normal and malignant cells was assayed by both ^{51}Cr-release cytotoxicity and immunofluorescence staining and showed similar results. Only immunofluorescence data are shown here.

CLL, chronic lymphocytic leukemia; cCLLa, common CLL-associated antigen; HCL, hairy cell leukemia; PBL, peripheral blood lymphocytes; PWM, pokeweed mitogen; PHA, phytohemagglutinin; CON-A, concanavalin-A; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; AMMoL, acute myelomonoblastic leukemia; APrML, acute promyelocytic leukemia; CML, chronic myelogenous leukemia.

*Reactive cells are reported as percentage (SEM) of 600 cells counted for each assay except negative controls, which are reported as range.

PBL, T cells, and B cells from normal subjects showed only background reactvity with the αCLL antibody. Likewise, normal thymus, spleen, and lymph node lymphocytes were equally unreactive, as were bone marrow aspirates from four patients with anemia of chronic illness (Table 4). To evaluate whether the cCLLa represented a B cell differentiation antigen not expressed on mature B cells, the αCLL antiserum was reacted against cells likely to express such differentiation antigens, including cord blood lymphocytes, and 3-, 5-, and 7-day cultures of mitogen-stimulated normal blood lymphocytes.[22] As shown, lymphoid cells from all ten source categories and marrow interface cells failed to express the CLL antigen. The possible expression of the cCLLa on malignant cells of other types of leukemias was also investigated. Leukemias studied were: cALLa-positive and cALLa-negative acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), acute myelomonoblastic leukemia (AMMoL), acute promyelocytic leukemia (AprML), and chronic myelocytic leukemia (CML) in blast crisis. As shown, none of the 32 leukemic patients examined expressed the cCLLa. Likewise, blood and lymph node lymphocytes from 14 patients with non-Hodgkin's lymphoma as well as lymph node lymphocytes and splenocytes from three patients with advanced Hodgkin's disease did not express the cCLLa antigen. Last, 14 lymphoblastoid B cell lines, 4 lymphoblastoid T cell lines, 1 fetal lymphoid cell line, and 1 pluripotent hematopoietic cell line also failed to express the cCLLa. Selective expression of the cCLLa described above has been confirmed by mouse monoclonal antibodies (Faguet and Agee, manuscript in preparation).

Isolation and partial characterization of the cCLLa. From 2.5×10^{10} CLL cells (B phenotype), 52 mg of membrane protein were recovered with a specific antigen activity of 0.1 U/μg as judged by cytotoxicity inhibition assay expressed as percentage of inhibition of 1 μg of αCLL IgG by 1 μg of membrane protein. Of this, 2.75 mg protein was recovered after passage through an αCLL-Sepharose affinity column. The immunoabsorbed-bound material had a specific antigen activity of 71 U/μg (710% cCLLa enrichment). Further fractionation by ion-exchange chromatography on a DEAE-cellulose column revealed three distinct peaks: Peak 1 had no activity at concentrations up to 150 μg per well; peak 2 consisted of 1.5 mg protein with cCLLa-activity of 156 U/μg protein (1,560-fold enrichment). Peak 3 consisted of a small component (0.8 mg protein) with low activity (2.7 U/μg protein). The specificity of peak 2 is shown by the fact that it did not inhibit lysis of B cells by the αB antiserum at concentrations up to 1,000 times that required for complete inhibition of αCLL-induced lysis of CLL cells. SDS-PAGE gels of peak 2 revealed a major band with an estimated mol wt of 69 kd both in the presence or absence of β-mercaptoethanol and heating (Fig 2). Two experiments served as controls: (a) Using the above procedure, B cell membranes prepared from 2.5×10^{10} B lymphocytes were applied to an αCLL-IgG-Sepharose affinity column; (b) an equivalent CLL membrane preparation was applied to an antihemoglobin A-IgG-Sepharose column equal in size to the αCLL-IgG-Sepharose affinity column. The immunoabsorbed material recovered from either column (0.56 mg and 0.84 mg of protein, respectively) showed no cCLLa activity at concentrations up to 150 μg of protein per well. In addition, further fractionation of these materials on a DEAE-cellulose column revealed only one peak (peak 1) with no cCLLa reactivity.

Further characterization of the cCLLa by monoclonal antibodies. The identity of the cCLLa was further ascertained as follows: Aliquots of 10^6 B-CLL cells enriched normal T and B lymphocytes were either preincubated with αCLL or not prior to exposure to a panel of monoclonal antibodies. Fresh cells were phenotyped as described in the Materials and Methods section. Cells preincubated with αCLL were assayed by indirect IFA using fluoresceinated goat anti-mouse or goat anti-rabbit antibodies to monitor mouse monoclonal and rabbit xenogeneic antibody binding, respectively. As shown in Table 1, the phenotype of fresh CLL cells and of fresh enriched circulating normal T and B lymphocytes demonstrated homogeneity of lineage. Fresh cells from each subset exhibited only background reactivity when exposed to J-5 and HTLV-1 antibodies (a generous gift from Dr D. Bolognesi, Duke University, Durham, NC). As expected, the LEU-1 antibody reacted with 88.3% of fresh CLL cells and with 92% of fresh T cells and showed background reactivity with fresh B cells. In contrast, the CLL-1 and αCLL antibodies reacted with 85.1% and 82.5%, respectively, of fresh CLL cells but did not react with either fresh normal T or B lymphocytes. The reactivity of LEU-1 with target CLL cells (88.3%) and with normal T lymphocytes (92%) was not altered by preincubation with αCLL (83.9% and 95.3%, respectively). In contrast, CLL-1 reactivity with target CLL cells (85.1%) was completely abrogated (2.9%) by preincubation with αCLL. That competitive binding to the target antigen by αCLL accounts for the abrogation of CLL-1 reactivity is supported by the use of goat anti-rabbit second antibody. Moreover, experiments in which preincubation of the CLL-1 with purified cCLLa completely abrogated its reactivity with target CLL cells demonstrate that the target antigen CLL-1 is indeed cCLLa (manuscript in preparation). Using a large panel of normal and neoplastic cells, CLL-1 reactivity has been demonstrated to parallel that of αCLL (Faguet and Agee, manuscript in preparation). As expected from their respective specificities for antigens other than the cCLLa, reactivity of the other antibodies of the panel was not altered by preincubation with αCLL and is shown in Table 1 as controls. The above observations strongly suggest nonidentity between the cCLLa and LEU-1 antigens and clearly demonstrate that the cCLLa is the common antigenic determinant for both CLL-1 and αCLL.

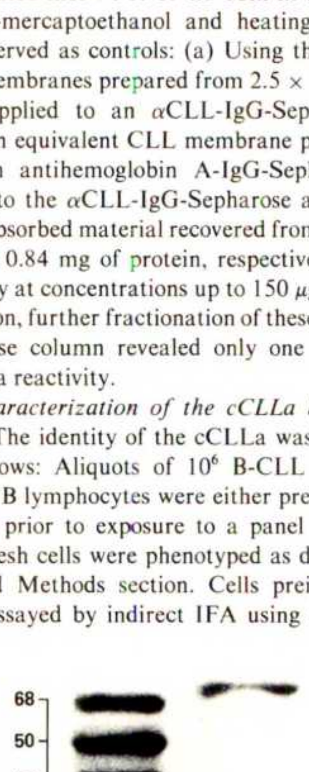

Fig 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of diethylaminoethanol (DEAE)-cellulose column peak 2 under reducing conditions (right lane). Mol wt markers (left lane) include: bovine albumin; IgG H-chain; ovalbumin; IgG L-chain; β-lactoglobulin; and lysozyme. Marker weights ($\times 10^{-3}$ daltons) are indicated.

DISCUSSION

The purpose of the present study was to explore the possible existence of distinct antigenic markers on CLL cells. Sequential absorption of the initial CLL antiserum with AB erythrocytes and HLA-matched peripheral blood lymphocytes resulted in a serum devoid of antibody activity against RBCs and T cells but retaining cytotoxicity against CLL cells and, to a lesser extent, B cells. Differential absorption with a panel of normal enriched B cells or CLL cells of B lineage suggested that the antiserum contained antibodies to an antigen found on 20% to 70% of B cells from ~50% of normal subjects and B-CLL patients and also antibodies against an antigen present on CLL cells but not on normal B lymphocytes. The different susceptibility of the two cell populations to antiserum αCLL/αB is attributed to two distinct antigens rather than to a different density of the same antigen on the two cell populations. This view is supported by the following observations: (a) distinct reactivity patterns after selective absorption with normal B or CLL cells; (b) persistence of anti-CLL activity after absorption with fivefold excess of B lymphocytes bearing free B antigen; (c) failure of autologous normal B lymphocytes to absorb anti-CLL activity, and (d) absence of cCLLa in normal B membrane preparations as demonstrated by chromatography studies and binding blocking experiments.

The cCLLa was expressed on most cells from all CLL patients studied but not on peripheral blood lymphocytes, enriched T and B cells from normal subjects, lymphocytes isolated from the thymus and spleen, or on other hematopoietic cells. However, these findings do not rule out the possibility that the antigen may be expressed by a minor subset of normal lymphocytes most susceptible to neoplastic transformation or that it is only transiently expressed early during differentiation, at a time when lymphocytes may be particularly susceptible to transformation and maturation arrest. This is consistent with the findings that CLL cells appear to be arrested in differentiation at an intermediate step between the pre-B and mature B lymphocyte stages.[23] The cCLLa was not detected on adult or fetal lymphoid cells or on cultured lymphoblastoid cell lines, in contrast to other xenoantisera generated against CLL.[6,22] In addition, retention of anti-CLL reactivity after absorption of αCLL with autologous residual normal B lymphocytes (fully histocompatible with respect to the normal antigens on the patient's leukemic cells) provides additional experimental evidence that CLL B cells express an antigen not detectable on autologous mature normal B cells. Unlike known differentiation antigens, this antigen was likewise not expressed on cord blood lymphocytes or during in vitro transformation of T and B lymphocytes using appropriate mitogens. This, and the fact that the cCLLa and LEU-1 are antigenically distinct, suggest that unlike the T cell differentiation antigen complex gp 65–71,[24-26] the 69-kd mol wt cCLLa may be more a reflection of the disease than a normal marker of lymphocyte differentiation unless the event occurs early at a stem-cell level. This is unlikely, however, because lymphoblasts from childhood and adult ALL failed to express the cCLLa. Moreover, because of the apparent common cell origin of B lymphocytes and myeloid cells in CML as judged by cytogenetics and enzyme markers,[27] some myeloid cells would be expected to express the cCLLa. Although such a predicted reactivity pattern has been observed with certain reported xenogeneic anti-CLL antisera that reacted with CML and AML cells,[12,13] none of the relatively few (N = 18) myeloid leukemias studied thus far expressed the cCLLa, nor did K562 cells, a pluripotent cell line derived from a patient with CMLBC.[28] The observation that lymphoma cells failed to express the cCLLa was unexpected, given the biologic and phenotypic similarities between these tissues and CLL. However, the number of cases studied thus far is small and both Hodgkin's tissues and non-Hodgkin's PBL are expected to have low if any cCLLa reactivity. Thus, recognition of a small subset of lymphoma patients with a cCLLa-positive clone requires further accrual of cases to our studies.

The clear demonstration of the cCLLa on malignant cells from nine patients with B-type HCL and three patients with PLL supports the view that CLL, PLL, and HCL are intermediary forms of lineage-related disorders[4] and suggests a common etiology for cCLLa-positive CLL, PLL, and HCL. In studies similar to those shown in Table 1, it was demonstrated that cCLLa is distinct from the HCL antigen recognized by monoclonal anti-HCL antibodies αHC1 and αHC2[29] (a gift from Dr D. Posnett, Rockefeller University, NY). Other monoclonal antibodies have been developed against normal and malignant T and B lymphocytes.[29-33] However, these antibodies also react with surface determinants shared by malignant and normal lymphoid cells, contrary to the restricted expression of the cCLLa recognized by αCLL and CLL-1 antibodies. The presence of HTLV-1 on CLL cells and its potential relationship to cCLLa were studied by binding and by cross-competition binding, respectively, using specific monoclonal antibodies against the major core proteins P19[34] and P24[35] and αCLL as blocking antibody in an IFA. Similar results were obtained using HCL cells as target cells (not shown). These findings and the observations that: (a) primary and cultured tumor cells of HTLV-1 positive malignancies are mature T cells[36,37]; (b) human cord blood exposed to HTLV-1 isolates infect T lymphocytes[38]; and (c) HTLV-1 sequences were absent in autologous B cells of patients whose neoplastic T cells contained HTLV-1 proviral sequences,[39] suggest that cCLLa-positive CLL, PLL, and HCL are not etiologically related to HTLV-1–induced T cell malignancies. However, our studies do not exclude an etiologic role for other retroviruses in B-lymphoid malignancies.

Monoclonal antibodies specific for the cCLLa and B cell lines expressing this antigen may be of clinical value: Detection of the cCLLa facilitates the diagnosis of a malignant lymphoid clone (manuscript in preparation) in a preclinical and potentially eradicable phase of its development. In addition, cCLLa-positive cell lines can be used to develop xenogeneic transplantation models (Faguet and Agee, manuscript in preparation) to ascertain the potential in vivo usefulness of cytolytic anti-CLL monoclonal antibodies and of their cytotoxic conjugates as immunotherapeutic agents.

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Nonrandom Involvement of the 12p12 Breakpoint in Chromosome Abnormalities of Childhood Acute Lymphoblastic Leukemia

By Susana C. Raimondi, Dorothy L. Williams, Thomas Callihan, Stephen Peiper, Gaston K. Rivera, and Sharon B. Murphy

We studied the presenting clinical and biologic features of 23 children with acute lymphoblastic leukemia (ALL) whose leukemic marrow karyotypes contained abnormalities involving the short arm of chromosome 12. Nineteen of the abnormalities were assigned to the 12p12 breakpoint. The median age of the children was 5 years (range 2 to 13 years) and their initial leukocyte counts ranged from 1,800 to 424,000/μL (median 30,000/μL). Twenty-one patients (91%) had common phenotype ALL (CALLA+, HLA-DR+), including three cases with a pre-B cell phenotype (CIg+). The remaining two cases were T cell in origin. The French-American-British (FAB) morphologic type of lymphoblastic leukemia was L1 in all cases but one. With a median follow-up of 11 months, four patients have relapsed and another failed induction therapy. The modal chromosome number in all cases was <50. Three distinct cytogenetic patterns, with apparently similar clinical manifestations, were noted: terminal deletions of chromosome 12 in 10 cases, apparently balanced reciprocal translocations in 6, and unbalanced translocations in 7. All translocations were between the 12p arm and different donor chromosomes except for chromosomes 7, 9, and 17, which participated twice. Only two patients had identical translocations: t(7;12)(q11;p12). This unusual variation in donor chromosomes and breakpoints suggests that translocations involving the 12p are specific with respect to only one member of the translocation pair, namely chromosome 12. The relatively high frequency of the 12p abnormalities in this study (10% of all completely banded cases seen over a 35-month period) warrants further investigation.

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SINCE THE THIRD International Workshop on Chromosomes in Leukemia, 1980 (TIWCL), chromosome findings have been recognized as an important independent prognostic factor in ALL.[1] Despite difficulty in obtaining banded chromosomes, several independent groups have reported data similar to the findings of the workshop for both adults and children.[1-4] Previously, we confirmed the prognostic importance of chromosome number (ploidy groups) in 136 consecutively studied children with ALL.[5] That study demonstrated superior treatment responses for children with >50 chromosomes and poorer outcomes in children with pseudodiploid karyotypes, regardless of other risk factors. In addition, data published by the TIWCL,[1] Bloomfield and co-workers,[3] and Williams and co-workers[6] have indicated that translocations generally confer a poor prognosis in ALL. As a result of these findings, increased attention has been paid to the role of genetic rearrangements in the pathogenesis and clinical course of ALL.

The TIWCL also reviewed the characteristics and clinical correlations of nonrandom structural chromosomal abnormalities including t(4;11), t(8;14), t(9;22), 14q+ and 6q−. Williams and co-workers[7] have described two additional nonrandom translocations, the t(1;19) specific for pre-B ALL and the t(11;14) specific for the E+ subset of T cell ALL, as well as the 12p12 abnormality found primarily in association with the common ALL immunophenotype. We present here additional cytogenetic data on the frequency and various forms of the 12p abnormalities (primarily at breakpoint 12) in 23 cases of childhood ALL, together with the clinical and biologic correlates of these changes. Previously reported for individual cases[2,8,9,10] and proposed as a distinct cytogenetic subgroup,[7] 12p abnormalities emerge as one of the most frequent abnormalities encountered in our cytogenetic studies of childhood ALL. The 12p12 breakpoint is of particular interest because of its recent association with the human cellular homolog of the Kirsten sarcoma gene (K-*ras* 2).[11,12]

MATERIALS AND METHODS

Patients

The study group consisted of 23 children with newly diagnosed ALL who were admitted to St Jude Children's Research Hospital between November 1981 and December 1984. Three patients (nos. 5, 16, and 17) were also studied at relapse. Each child received modern multidrug systemic therapy and central nervous system (CNS) prophylaxis.[5,13]

Morphologic and Cytochemical Studies

Following informed consent, bone marrow aspirates obtained from the iliac crest were stained with Wright's stain and examined for cell morphology and differential counts. Morphologic classification followed criteria of the French-American-British (FAB) system.[14] The cytochemical stains used in light microscopic examinations included: myeloperoxidase, Sudan black B, periodic acid-Schiff (PAS), and α-naphthyl butyrate esterase (ANBE).[15]

Immunophenotyping

All patients were tested for their blast cell phenotype before treatment with use of standard methods for detection of surface and cytoplasmic markers characteristic of the lymphoid lineage and degree of differentiation.[16] Bone marrow aspirates were collected in heparin, and the mononuclear cells were separated on a Ficoll-

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Table 1. Clinical, Biologic, and Cytogenetic Findings Associated With the 12p Abnormalities

Patient No.	Age/Sex	Leukocyte Count (μL)	FAB Type	Immunophenotype	No. of Metaphases Completely Analyzed	Metaphases with 12p Abnormalities (%)	Modal No. of Abnormal Clone	12p Abnormality	Additional Chromosome Abnormalities
Group I									
1	2/M	122,000	L1	Common	8	36	46	del(12)(p12)	—
2	9/F	424,000	L2	T cell	21	48	46	del(12)(p12)	—
3	8/F	14,000	L1	Common	33	24	46	del(12)(p12)	—
4	4/M	1,800	L1	Common	18	89	46	del(12)(p12)	Variation due to clonal evolution
5	9/F	154,800	L1	Common	5	100	46	del(12)(p12)	del(9)(p21)*
6	4/F	5,700	L1	Common (pre-B cell)	18	89	47	del(12)(p12)	−13, +mar, +mar
7	6/M	8,900	L1	Common (pre-B cell)	7	100	46	del(12)(p12)	del(8)(q21.2q21.3),del(9)(p21),* der(16),t(16;?)q24;?)
8	2/F	34,300	L1	Common	8	75	47	del(12)(p12)	+16
						25	47	del(12)(p12)	−13, +16, +mar
9	3/F	5,900	L1	Common	8	50	48	del(12)(p11)†	+11, −17, +mar, +mar
10	3/F	11,300	L1	Common	14	56	46	del(12)(p11)†	
						43	46		t(7;12)(p22;q12)
Group II									
11	5/M	79,000	L1	Common	12	100	46	t(1;12)(q25;p12)	—
12	11/M	2,200	L1	Common	16	50	46	t(3;12)(q12;p12)	del(6)(q15)
						50	46		del(6)(q15)
13	4/F	43,500	L1	Common	10	100	46	t(8;12)(p22;p13)†	—
14	2/M	41,400	L1	Common (pre-B cell)	15	93	46	t(9;12)(p21;p12)*	—
15	5/F	8,200	L1	Common	6	83	47	t(10;12)(q22.1;p12.3)	+21
16	6/F	4,100	L1	Common	12	91.5	47	t(12;17)(p12;q12)	+21,inv(6)(p11q15)
Group III									
17	6/F	17,000	L1	Common	20	60	49	der(12),t(2;12)(q14;p12)	+X, +del(16)(q13), +del(16)(q13)
						40	49	der(12);t(2;12)(q14;p12)	+X, +del(16)(q13), +del(16)(q13), del(7)(p11)
18	2/M	30,000	L1	Common	8	38	46	der(12),t(7;12)(q11;p12)	−7, +mar,del(6)(q14)
						22	45	der(12),t(7;12)(q11;p12)	−7,del(6)(q21)
19	2/M	52,600	L1	Common	23	40	46	der(12),t(7;12)(q11;p12)	−7, +21,del(1)(q32)
20	4/M	132,000	L1	Common	6	50	45	der(12),t(9;12)(9qter→?9cen ::12p12→12qter)	−9,del(X)(q26)
21	13/F	85,700	L1	T cell	15	70	45	der(12),t(12;17)(p11;q11)†	−17,inv(5)(p13q14),del(10)(q22), del(11)(q22)
22	12/F	22,400	L1	Common	12	80	46	der(12),t(12;?)(p12;?)	−X, +16,del(9)(p21)*
						20	46	der(12),t(12;?)(p12;?)	−X, +16,del(1)(q42),del(9)(p21)*
23	7/F	40,400	L1	Common	14	100	46	der(12),t(12;?)(p12;?)	−19, +del(7)(p15),inv(5)(p13p15), ?t(8;19)(8qter→8cen::19cen→19qter)

*Most consistent additional abnormality.

†Indicates the cases that had the 12p breakpoint not assigned to 12p12.

Hypaque gradient. Only samples containing >50% blasts were studied.

Cytogenetic Studies

Bone marrow samples from 21 patients and peripheral blood for 24-hour cultures in two patients (patients 1 and 18) were obtained at the time of diagnosis and processed according to the method of Williams and co-workers,[17] which incorporates numerous alterations of standard techniques. In brief, ~0.1–0.2 mL of heparinized bone marrow were collected in tubes containing 8 mL of RPMI 1640 with L-glutamine, antibiotics, and 30% fetal calf serum (FCS). The bone marrow cells were processed immediately following a 25-minute incubation in Colcemid (final concentration 0.06 μg/mL). Cells were rinsed twice in Hanks' balanced salt solution (HBSS). They were then exposed to hypotonic treatment (KCL 0.075 mol/L), for a total of 32 minutes at room temperature, including periods of mixing, standing, and centrifugation. The cells were then fixed in methanol-acetic acid (3:1, vol/vol) for 15 minutes. Slides were prepared using a flaming technique. The 24-hour unstimulated peripheral blood cultures were processed as described for bone marrow. Slides were allowed to age for 0 to 7 days. Metaphase preparations were G-banded by a modification of the trypsin method of Seabright[18] and chromosomes were identified and assigned according to the International System of Human Cytogenetics Nomenclature.[19]

RESULTS

Clinical and Biologic Characteristics

The presenting clinical features of the 23 children with an abnormality of the p arm of chromosome 12 are summarized in Table 1 according to the karyotypic complexity of each case as follows: terminal deletion (group I), apparently balanced reciprocal translocation (group II), and unbalanced translocation (group III) of known or unknown origin. There was no obvious pattern of clinical characteristics distinguishing these subsets of patients from the entire group seen during the same 35-month period. Leukocyte counts ranged from 1,800 to 424,000/μL (median, 30,000/μL), and ages ranged from 2 to 13 years (median 5). The ratio of males to females was 14:9. Patients 13, 14, and 20 had initial CNS involvement, but a mediastinal mass was not present in any of the subjects. With a median follow-up of 11 months (range 2 to 35 months), four patients (nos. 5, 14, 16 and 17) have relapsed, and another patient (no. 24) failed induction therapy.

The morphologic type of lymphoblastic leukemia was L1 in all cases except number 2, which was classified as L2. None of these patients' blast cells reacted with myeloperoxidase or Sudan black B, but PAS reactivity was noted in three cases (nos. 1, 2, and 5). Ten of the 24 marrow specimens were stained for ANBE, with occasional positive granules seen in three cases (nos. 14, 16, and 23).

Immunophenotypic analysis with a large panel of monoclonal antibodies disclosed CALLA+, HLA-DR+ blast cells in 21 (92%) of the 23 cases, consistent with differentiation in the B cell pathway.[20] Cytoplasmic immunoglobulin was present in three of the cases, indicative of a pre-B cell type. Two of the 25 patients had membrane markers denoting a T lymphocyte lineage. Blast cells from patient 2 were positive for the pan T cell antigens T9, T10, T6, and T4 corresponding with early to midthymocyte stages, whereas those from patient 21 had an early thymocyte phenotype as indicated by positivity for the T9 and T10 antigens.

Cytogenetic Features

The frequency of 12p abnormalities was ~10%, or 23 of 241 patients with completely banded chromosomes who were seen over a 35-month period. The cytogenetic findings are presented in detail in Table 1 and illustrated in Figs 1 through 3. In the individual cases, the 12p abnormality was found in 24% to 100% of all completely analyzed metaphases. However, such changes were also observed in metaphases that were not suitable for complete analysis, indicating that some cases were under-represented in this study. The majority of cases had 46 chromosomes, with a lower modal number in only two instances and hyperdiploidy (47 to 49) in six.

Group I (terminal deletion). Patients 1 through 8 (Table 1) had a terminal deletion of the p arm of chromosome 12 at band 12; examples are illustrated in Fig 1A through D. Three of these cases had the del(12)(p12) as their sole abnormality. The other five cases contained additional chromosome abnormalities that were either structural or numerical but apparently did not involve translocations; an exception was noted for patient 7, who had a derivative chromosome 16

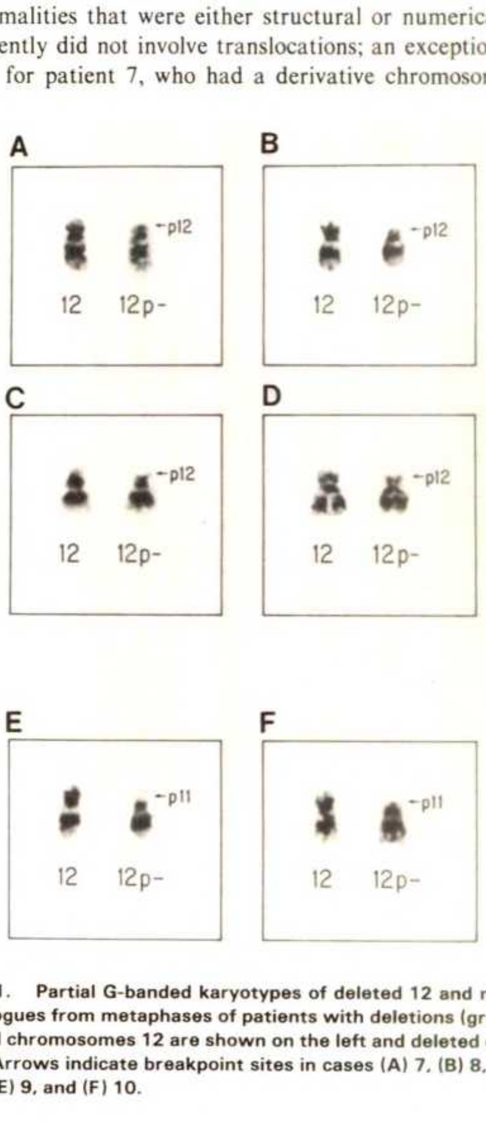

Fig 1. Partial G-banded karyotypes of deleted 12 and normal homologues from metaphases of patients with deletions (group I). Normal chromosomes 12 are shown on the left and deleted on the right. Arrows indicate breakpoint sites in cases (A) 7, (B) 8, (C) 6, (D) 5, (E) 9, and (F) 10.

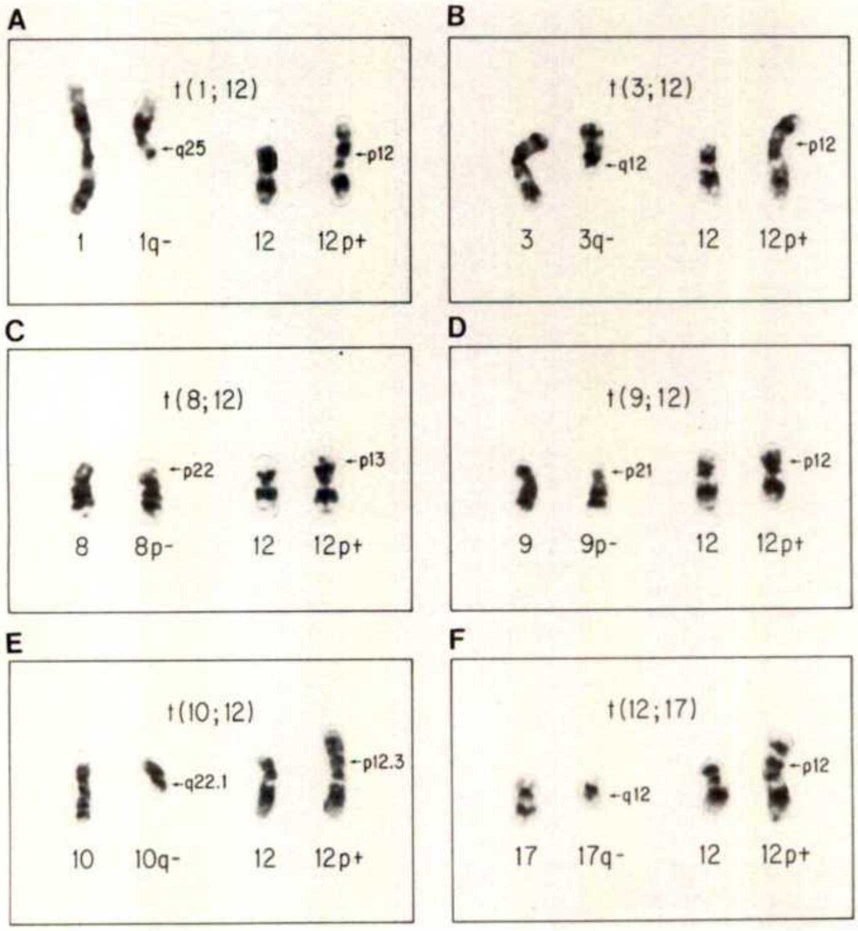

Fig 2. Representative G-banded partial karyotypes of rearranged chromosomes and homologues from metaphases of patients with apparently balanced reciprocal translocations (group II). Panels A through F illustrate changes in patients 11 through 16. The normal chromosomes are shown on the left and the rearranged ones are shown on the right of each pair. Arrows indicate breakpoint sites.

with added DNA of unknown origin. Patients 9 and 10 also presented with a terminal deletion of the p arm of chromosome 12 but at band 11; examples are illustrated in Fig 1E and F. In addition, patient 9 had a complex karyotype with markers of unknown origin. Two apparently unrelated pseudodiploid lines were observed in blast cells from patient 10. Although both independent lines involved chromosome 12, the breakpoints were at different sites in the two stem-lines (p11 and q12).

Group II (apparently balanced reciprocal translocation). A second group was defined by apparently balanced reciprocal translocations between the p arm of chromosome 12 at band 12 and five different chromosomes, as shown in Fig 2A and B and D through F. Lymphoblasts from patient 13 contained an apparently balanced reciprocal translocation between chromosomes 8 and 12, this being the only case with a 12p13 breakpoint (Fig 2C). The karyotypes of the entire group were generally not complex.

Group III (unbalanced translocation). The third group was characterized by unbalanced translocations and complex karyotypes with both numerical and structural abnormalities. To facilitate the description, this group was subdivided into cases with DNA of known and unknown origin translocated to the 12p arm.

Genetic material of known origin was translocated to 12p in five cases (patients 17 through 21, Table 1) (Fig 3A through E). In patient 17, a derivative 12 chromosome was the result of an unbalanced translocation between chromosomes 2 and 12, but with two normal chromosomes 2 also present (Fig 3A). Two related abnormal lines were present due to clonal evolution. The 12p+ in patients 18 and 19 (Fig 3B and C) resulted from an unbalanced translocation between chromosomes 7 and 12. Both cases had identical der(12) chromosomes but different additional chromosome abnormalities. This was the only translocation that was observed twice among all patients with 12p abnormality.

Figure 3D depicts the translocation between chromosomes 9 and 12, with the exact breakpoint in chromosome 9 being uncertain. In all the metaphases containing the der(12), t(9;12), the derivative 9 chromosome was lost, resulting in an unbalanced translocation. Patient 21 (Fig 3E) also had translocated material of known origin (chromosome 17) to chromosome 12p but with the breakpoint assigned to p11. In all the metaphases with the der(12),t(12;17), the small derivative 17 chromosome was missing resulting in an unbalanced translocation. An additional translocation between chromosomes 12 and 17 was found in patient 16 (Fig 2F) but with different breakpoints.

Genetic material of unknown origin was translocated to 12p12 in two cases (patients 22 and 23). In the first (Fig 3F) the 12p+ was present in both the stem-line and a side-line. In the other case (Fig 3G), additional chromosome abnormalities included a possible second translocation between chromosomes 8 and 19.

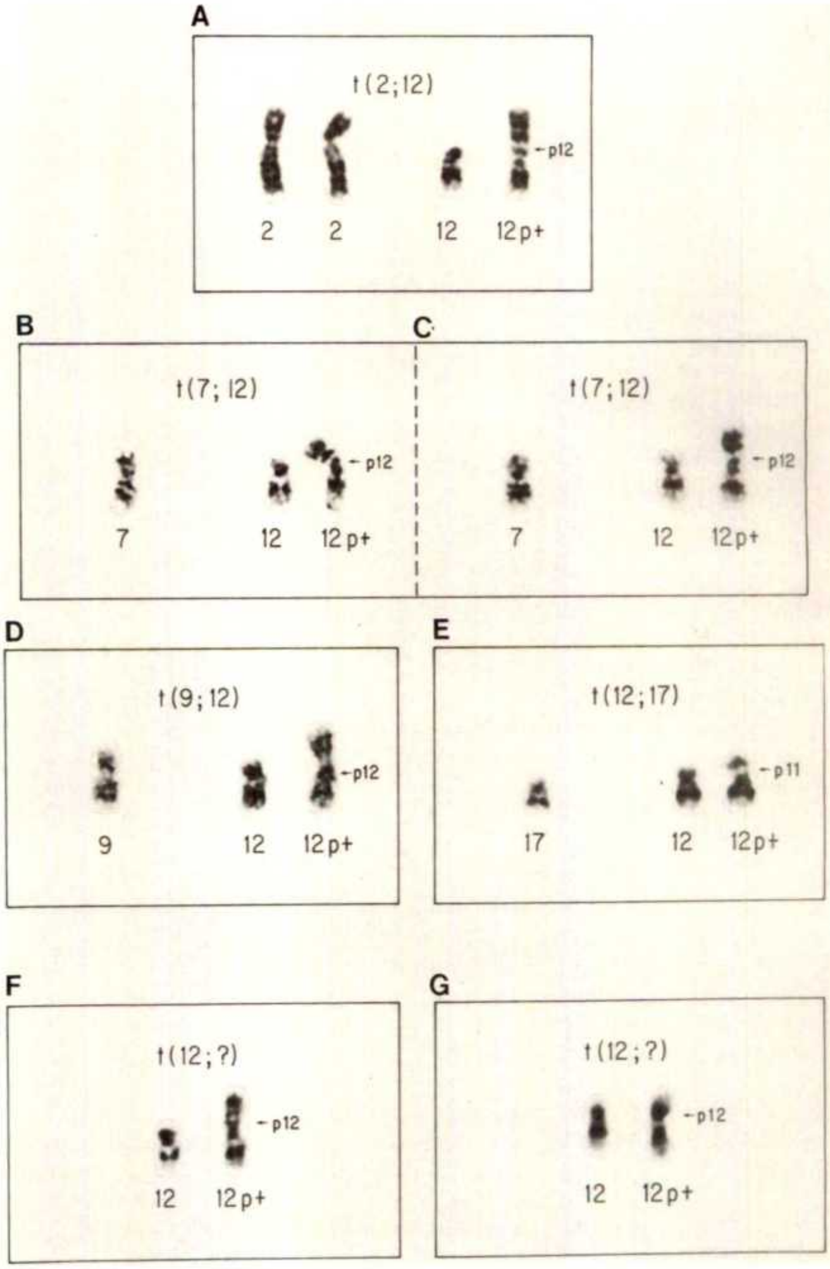

Fig 3. Representative G-banded partial karyotypes of rearranged chromosomes and homologues (when applicable) from patients with unbalanced translocations (group III). Translocated genetic material of known origin: panels A through E (patients 17 through 21) or unknown origin: panels F through G (patients 22 and 23). The normal chromosomes are shown on the left, and the rearranged ones are shown on the right. Arrows indicate breakpoint sites.

General cytogenetic findings. Most of the additional chromosome abnormalities involved structural changes that were varied and inconsistent, except for a deletion of chromosome 9 with breakpoints at p21 (three patients) and an extra chromosome 21 (three non-Down syndrome patients) (Table 1). In contrast to groups I and II, group III had karyotypes of great complexity. In six patients (nos. 4, 8, 12, 17, 18, and 22), there was evidence of karyotypic evolution at diagnosis, suggested by the presence of additional abnormalities not observed in the stem-line. Patient 10 had two apparently independent clones. Although an abnormality of chromosome 12 was involved in both clones, the breakpoint in one was at p11, and in the other at q12.

Patients 16 and 17 were also studied cytogenetically at the time of their first hematologic relapse. Karyotypes of patient 16 determined at diagnosis and relapse were identical; however, patient 17 received a bone marrow transplant after her first relapse but relapsed again 5 months later. At that time, two abnormal lines were present containing markers found at diagnosis, as were additional markers, indicating clonal evolution and progressive growth of the tumor cell population.

DISCUSSION

Reports of abnormalities involving the short arm of chromosome 12 have been sporadic.[21] We have reported four childhood cases[7] and one each has been reported by Whang-Peng and colleagues,[2] Prigogina and colleagues,[8] Morse and colleagues,[9] and Kowalczyk and colleagues.[10] Previous individual reports of the 12p12 abnormality in adults with ALL at the time of diagnosis include only a few cases.[22-25] Recent observations indicate that this abnormality can also be associated with myeloid leukemia.[25,26] In our series of patients with acute nonlymphocytic leukemia, the 12p abnormality was found in only 2 of >100 cases studied. The limited number of children with ALL in earlier studies has precluded estimates of the prevalence of this chromosomal change. From results of the present study, it appears that 12p abnormalities comprise a major cytogenetic group accounting for ~10% of all newly diagnosed patients with this disease.

The relatively low frequency of 12p deletions previously reported most likely relates to the subtlety of this abnormality (Fig 1) and to the quality of sample preparations. The translocation of different chromosomes to the p arm of chromosome 12 (Figs 2 and 3) can result in 12p abnormalities that are more easily detected than simple deletions. Using improved cytogenetic techniques specifically suited to ALL chromosomes, one might expect to find more instances of this chromosomal change in large series of well-studied childhood leukemia patients.

Previous accounts of 12p abnormalities included cases representative of each of the subgroups presented in this study, but recognition of the natural subgrouping of deletions and balanced and unbalanced translocations was not possible because of the limited number of cases evaluated. Future studies may establish that the missing part of the deleted chromosome 12 is translocated to other chromosomes in the complement, although present techniques are inadequate to substantiate this idea. Of the 13 translocations evaluated here, 11 had a breakpoint at 12p12, and one breakpoint each at p11 and p13. In most instances, the abnormal chromosome 12p was involved with different donor chromosomes (1–3, 7–10, and 17), suggesting that breakage in the 12p arm may be a critical event in malignant transformation or in providing the cells with a proliferative advantage.

Because most donor chromosomes in the 12p group of abnormalities are different or, if similar, have different breakpoints, one can argue that the subgroups do not represent specific cytogenetic categories but rather are unique. Nevertheless, the subdivisions we recognized should help to distinguish among major karyotypic differences and should prove useful in future comparative studies.

The only identical translocation observed in this study was a t(7;12)(q11;p12) in two patients, a finding not previously reported. Kaneko and co-workers[24] reported an apparently balanced translocation, [t(12;17)(p13;q12)], that was similar to the abnormality we observed in patient 16, [t(12;17)(p12;q12)], except for a slight difference in the assignment of the breakpoint on chromosome 12, which could have resulted from the different banding techniques used. Patient 21 also had a t(12;17), but it was unbalanced and formed a 12p+, with the 17q arm attached to 12p and the 17p being lost. There were two cases in which other translocations had the same donor chromosomes: a balanced t(9;12) in patient 14 and an unbalanced t(9;12) in patient 20. Hence, our findings indicate a variable composition of the karyotypes in the 12p ALL group. Nevertheless, with future studies of additional well-banded cases, it is likely that additional identical cytogenetic subgroups will be identified. Other chromosome abnormalities most frequently found in our patients were 9p- and +21. A similar 9p deletion was reported previously.[9] Furthermore, Kaneko and co-workers[25] and Kowalczyk and Sandberg[27] suggested that most 9p deletions are found in T cell ALL. All three patients with 9p− in our study had a common ALL immunophenotype. The extra chromosome 21 is a common consistent addition in ALL.[1]

Multiple lines at the time of diagnosis appears to be more commonly observed in ALL than in ANLL.[24] Although at present the clinical and prognostic significance of multiple abnormal lines at diagnosis is unknown, we noted that patients 4, 8, 12, 17, 18, and 22 had related abnormal lines, apparently derived from the same clone. Only patient 10 had two stem-lines, which appeared to be unrelated and to be derived from independent clones. Even though chromosome 12 was involved in both clones, the breakpoints affected the p and q arm independently. Thus, evidence of clonal evolution at the time of diagnosis was found in ~30% of all cases with the 12p abnormalities. This is slightly higher than the 22% reported by Kaneko and co-workers[24] in a consecutive series of 31 pediatric and 19 adult cases with ALL and the 26% in 116 cases included in our current ALL protocol (D. L. Williams, unpublished observations, 1985). Similar karyotypic instability at diagnosis has been reported by others,[22–24] and could indicate a more advanced stage of the disease.

It has been observed that the assigned loci of some human cellular oncogenes correlate with specific chromosomal rearrangements in certain malignancies. The nonrandom 12p12 abnormality reported here thus seems important in view of the recent assignment of the c-Kirsten-*ras* 2 oncogene to 12p12 and q24.2.[11] Furthermore, Popescu and co-workers mapped this oncogene to 12p11.1-12.1 by in situ molecular hybridization.[12] Because some oncogenes may be altered in cancer cells as a result of chromosomal rearrangements, particularly translocation,[28–30] it is believed that each of those recurrent chromosomal changes reflect specific alterations at the molecular level that ultimately trigger the development toward malignancy. Molecular studies of our 12p12 ALL cases are in progress in our institution.

The clinical significance of our findings is not yet known because the follow-up time for many cases has been too short to permit assessment of treatment responses. Even so, from previous findings[1,3] and those of our most recent analysis[6] in which a translocation in ALL was found to confer a poor prognosis, it is likely that patients in groups II and III, both containing translocations, are at higher risk for treatment failure. Group III (unbalanced translocations) had a slightly higher median leukocyte count and by far the most complex karyotypes of any of the three cytogenetic groups we identified. Determination of whether children with 12p abnormalities have different risks of treatment failure, either among themselves or by comparison with other identifiable cytogenetic groups of patients with ALL, requires study of a larger series of patients with such changes.

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Metabolism of Ara-C by Blast Cells From Patients With ANLL

By Douglas D. Ross, Bruce W. Thompson, Christopher C. Joneckis, Steven A. Akman, and Charles A. Schiffer

The dose–response relationship between extracellular concentration of cytosine arabinoside (ara-C) and intracellular formation of the putative active metabolites of ara-C [ara-C incorporation into DNA and intracellular pools of ara-C in triphosphate form (ara-CTP)] was investigated in blast cells obtained from patients with acute nonlymphocytic leukemia (ANLL) by exposing these cells in vitro to 10, 100, or 1,000 nmol/L of ara-C. We studied 23 untreated patients who subsequently achieved complete remission (CR) with a regimen using daunorubicin and conventional doses of ara-C (ara-C–sensitive group), and 30 patients judged to be ara-C–resistant either by failing initial induction therapy (16 patients) or by having relapsed on an ara-C–containing maintenance regimen (14 patients). In both patient groups, ara-C incorporation into DNA and intracellular ara-CTP both displayed statistically significant increases in response to increasing extracellular concentrations of ara-C ($P = .0001$ in both cases), with the rate of increase of ara-CTP greater than that of ara-C incorporation. Moreover, blast cells from all patients, even those who were most clinically resistant to ara-C, were able to form ara-CTP and to incorporate ara-C into DNA. Each tenfold increment in extracellular ara-C concentration caused an 8.5-fold increase in ara-CTP, but only a 3.6-fold increase in ara-C incorporation into DNA. Thus, the efficiency of incorporation of ara-C into DNA (defined as the ratio of ara-C incorporation to ara-CTP pools) decreased by 58% with each tenfold increment in the extracellular concentration of ara-C ($P < .0001$), presumably as a result of the inhibitory effect of ara-CTP on DNA polymerase. Using an analysis of covariance, modest differences were found in the levels of the ara-C metabolite variables in the ara-C–sensitive group as compared with the resistant group. However, because there was considerable overlap in ara-C metabolite formation among the patient groups, it was not possible to predict clinical outcome by these in vitro assessments of ara-C metabolism.

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THE ANTIMETABOLITE ara-C is one of the most effective agents currently available for the treatment of ANLL in adults. When combined with the anthracycline antibiotic daunorubicin, high remission induction rates have been reported for previously untreated patients.[1,2] Unfortunately, most patients eventually relapse and die of leukemia despite maintenance or continued therapy with ara-C–containing regimens, suggesting the emergence or expansion of ara-C–resistant leukemic cell populations.

Considerable evidence has accumulated concerning the mechanism of ara-C cytotoxicity to established leukemic cell lines. After entering the cell, ara-C must be converted to the triphosphate form (ara-CTP) by a series of kinase enzymes.[3-5] Ara-CTP is considered to be the active metabolic form of ara-C and is a powerful inhibitor of DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7), competitive with the natural substrate dCTP.[6-8] Although inhibition of DNA polymerase would surely retard cell growth, inhibition of DNA synthesis per se is not necessarily a cytotoxic event. It has been recognized that ara-C is a weak substrate for DNA polymerase and that small amounts are incorporated into the DNA molecule. In tissue culture systems, incorporation of ara-C into DNA correlates directly with the degree of cytotoxicity.[9] Indeed, recent studies in HL-60 cells[10] of the relationship among ara-CTP pools, formation of ara-C(DNA), and clonogenic survival demonstrated that incorporation of ara-C into DNA was the single most powerful predictor of cell killing by ara-C.

Various biochemical mechanisms of cellular resistance to ara-C have been recognized in cultured cells or in experimental animal tumor models. Cells have been identified with high levels of cytidine deaminase (EC 3.5.4.5) or dCMP deaminase (EC 3.5.4.b) which convert ara-C or ara-CMP to the inactive ara-U or ara-UMP metabolites, respectively.[11] Cells deficient in the nucleoside kinase enzyme (deoxycytidine kinase, EC 2.7.1.74) have been identified,[3,12] which are resistant because of inability to phosphorylate ara-C. Other cells have been found to be resistant to ara-C by their high intracellular levels of dCTP, which inhibit ara-C phosphorylation and which competes with ara-CTP for DNA polymerase.[13,14] A DNA polymerase insensitive to inhibition by ara-CTP or one that fails to recognize ara-CTP as a substrate is another means by which cells can become resistant to ara-C.[15,16] A DNA repair enzyme activity has been described recently that removes ara-C residues from DNA.[17] Thus, enhanced intracellular activity of such an enzyme may be a mechanism of cellular resistance to ara-C. Finally, increased dephosphorylation or intracellular catabolism of ara-CTP offers a potential mechanism of cellular ara-C resistance.[18]

Most of the cellular mechanisms of ara-C resistance mentioned above have been identified in ara-C–resistant cells in culture. However, at present, little data exist as to the mechanism(s) operative in blast cells from leukemia patients clinically resistant to ara-C. Intracellular kinase and deaminase studies have failed to predict clinical outcome,[19,20] as have studies of cellular ability to form ara-CTP,[21,22] although the ability of blast cells to retain intracellular ara-CTP

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following in vitro exposure to ara-C has been reported to correlate with remission duration in those patients who subsequently achieved a complete remission.[21,23] More recently, the intracellular pharmacokinetic studies of Plunkett and co-workers[24] suggest that retention of ara-CTP may be a determinant of response to therapy with high-dose ara-C.

Of the mechanisms of cellular resistance to ara-C outlined above, all should be manifested either by diminution in the steady-state intracellular concentration of ara-C, diminution of ara-C incorporated into DNA, or both. It should be noted that those mechanisms which involve enhanced cellular catabolism of ara-CTP and/or enhanced excision and repair of ara-C–induced DNA damage may only become apparent by observing the intracellular half-life ($t^{1}/_{2}$) of ara-CTP (ara-CTP retention) or of retention of ara-C incorporated into DNA following exposure of cells to ara-C.

In the present studies, we measured intracellular steady-state concentrations of ara-CTP and ara-C incorporated into DNA in blast cells from patients with ANLL exposed to varying concentrations of ara-C in vitro, in an effort to understand the dose–response relationship between ara-C and formation of its metabolites in ANLL blast cells, and to determine if differences in these ara-C-metabolic parameters and dose–response can be detected between groups of patients clinically sensitive and resistant to conventional doses of ara-C. Extracellular concentrations of 10, 100, and 1,000 nmol/L of ara-C were used, since 100 to 1,000 nmol/L approximates the steady-state plasma level of ara-C attained during conventional-dose continuous infusion of ara-C.[25]

MATERIALS AND METHODS

Patient selection and characteristics. In all patients, the diagnosis of ANLL was established on the basis of microscopic examination of Wright's-stained specimens of bone marrow, assisted by examination of specially stained specimens [α-napthyl esterase, chloroacetate esterase, terminal deoxynucleotidyl transferase (TdT), Sudan black, periodic acid-Schiff]. These studies were performed between August 1982 and January 1985. The median age of the patients studied was 52 years, with 43% females and 57% males. This approximates the overall demographics of ANLL patients treated at our center. Patients were subdivided into three groups based on their clinical response to ara-C–containing regimens: complete remission (CR) group (ara-C–sensitive patients), no response (NR) group (ara-C–resistant patients); and relapsed (REL) group (ara-C–resistant patients).

Complete remission group. The complete remission (CR) group comprised 23 patients who were untreated at the time of study who subsequently achieved CR with induction therapy consisting of daunorubicin (45 mg/m^2/d; days 1, 2, and 3) and ara-C (100 or 200 mg/m^2/d, days 1 through 7 by continuous intravenous infusion). All but four of the 23 patients in this group have had remission durations >6 months (overall range 2 to 19+ months), and 12 patients (52%) remain in CR at the present time. All patients received ara-C–based maintenance or consolidation regimens. The projected median duration of CR (Kaplan-Meier) for this group is 11 months. All marrows studied were obtained before any treatment was given.

No response group. The no response (NR) group (ara-C–resistant patients) comprised 16 patients who were either untreated at time of study (13 patients) or previously treated (three patients), but who failed to attain remission with an ara-C–containing regimen at any time during the course of their disease. For the previously treated patients, marrow was collected for study after a suitable period elapsed from the last treatment attempt to allow marrow recovery from that treatment (2 to 3 weeks). All patients in the NR group received an initial induction regimen identical to that described for the CR group above. All patients in this group survived their induction attempt and were fully evaluable for response to the ara-C containing regimen.[26]

Relapsed patient group (ara-C–resistant patients). Fourteen patients were studied at time of relapse. Seven patients in this group relapsed while on an intensive ara-C–containing maintenance regimen, and marrow was obtained for these studies prior to further treatment with investigational agents. The other seven patients had a previous CR but had relapsed and failed to respond to an ara-C–containing reinduction regimen. Of the REL group, 7 were in first relapse, 4 were in second relapse, 2 were in third relapse, and 1 was in fourth relapse at the time of study.

No patient was studied during therapy or within 2 weeks of the completion of a chemotherapeutic regimen. The data presented are from 53 different patients. Thus, the patients within a response category are unique to that category. In no case was a patient included in different response categories nor was any patient included twice in the same category.

Bone marrow collection and preparation. Bone marrow (4 mL) was aspirated from the posterior iliac crest of patients using 0.1 mL of preservative-free heparin (1,000 U/mL) as an anticoagulant. Informed consent for marrow collection for this study was obtained as reviewed and approved by the University of Maryland at Baltimore Human Volunteers Institutional Review Board. Marrow was placed in a 15-mL sterile conical centrifuge tube (Costar Plastics, Cambridge, Mass) and then diluted with an equal volume of RPMI 1640 medium (without serum). Marrow cells were dispersed by gentle pipetting and 5 mL of Ficoll-Hypaque ("LSM", Litton Bionetics, Inc) were layered under the marrow suspension. Mononuclear cells were collected at the Ficoll-Hypaque and medium interface after centrifugation (400 g for 40 min, 4 °C).[27] RBCs contaminating the mononuclear cell fraction were lysed by exposure to 0.74% NH_4Cl, following which the partially purified marrow mononuclear cells were washed once with ice-cold RPMI 1640 medium (without serum). Cells were resuspended in a volume of growth medium [RPMI 1640 medium containing 10% vol/vol heat-inactivated (56 °C for 30 min, fetal calf serum (FCS)] sufficient to achieve the desired final cell concentration (~0.8 to 1.2×10^6 cells per milliliter). This procedure yields mononuclear cells of which 80% to 100% have the morphologic appearance of myeloid blast cells when viewed by light microscopy. Viability (trypan-blue dye exclusion) was routinely 80% to 100%. Average blast cell yield from the marrows studied was ~2×10^8 cells.

Blast cell culture and exposure to ara-C or thymidine (dThd). Cells in growth medium (cell concentration, constant within each experiment, ranged from 1 to 2×10^6 cells per milliliter among experiments) were aliquoted into sterile 13 × 100-mm borosilicate glass culture tubes (1.8 mL per tube), and the appropriate amount of ^{3}H-ara-C or ^{3}H-dThd was then added in a volume of 0.2 mL of culture medium to achieve the final desired concentration. Stock 5-^{3}H-ara-C (15 Ci/mmol) was obtained from Amersham/Searle, Inc (Arlington Heights, Ill). The final concentrations in culture/specific activities used were 10 nmol/L (10 Ci/mmol), 100 nmol/L (5 Ci/mmol) and 1,000 nmol/L (1 Ci/mmol). 5-Methyl ^{3}H-dThd (New England Nuclear, Inc, Boston, specific activity 6.7 Ci/mmol) was added to the appropriate culture tubes to achieve a final concentration of 300 nmol/L. Cultures were incubated at 37 °C, 7.5% CO_2, for 22 hours, after which ara-C incorporation into

DNA, dThd incorporation into DNA, and intracellular ara-CTP was determined by methods described below. Zero-time "background" tubes were prepared by incubating cells in the absence of isotope for the 22-hour period and then adding the appropriate amount of ^{3}H-ara-C or ^{3}H-dThd after cooling the cells on ice for 15 minutes. Degradation of ara-C in culture medium alone after 22-hour incubation was minimal. Degradation of ara-C in the blast cell culture ranged from 23% to 37% for 10 nmol/L of ara-C, 11% to 29% for 100 nmol/L of ara-C, and 1% to 22% for 1,000 nmol/L of ara-C, using high-performance liquid chromatography (HPLC), as described previously.[28] The major breakdown product was ara-U. We intentionally avoided the use of cytidine deaminase inhibitors in these studies, since the objective of the experiments was to assess the overall ability of patient-derived blast cells to metabolize ara-C, including the effects of intracellular degradation of ara-C. Incorporation of ^{3}H-dThd into DNA was linear throughout the 22-hour period of incubation.

Determination of intracellular ara-CTP pool size. Following incubation, cultures were chilled on ice for 15 minutes, and cells were collected by sedimentation (400 g for 10 minutes, 4 °C), washed twice with ice-cold phosphate-buffered saline (PBS),[29] and then extracted twice with 200 μL of 60% methanol in H_2O, at 30 °C × 10 minutes. The two methanol extracts of each culture were combined; debris was removed by sedimentation (13,000 g for 10 minutes, 4 °C). Methanol extraction was used since we find[28] that ara-CTP is stable for at least 2 months in 60% methanol at −20 °C. Ara-CTP content of the methanol extracts was determined as reported previously[28] by injecting 80 μL of the extract, along with appropriate internal standards, into a model 3500B HPLC (Spectra-Physics, Santa Clara, Calif) equipped with a 4.6 mm × 25 cm Partisil SAX/10 anion exchange column (Whatman Inc, Clifton, NJ) and a 2.1 mm × 7 cm guard column filled with anion exchange packing. Samples were eluted with a mobile phase of KH_2PO_4 (500 mmol/L, pH 4.6) under isocratic conditions with a flow rate of 1.52 mL/min. Fractions (0.5 mL) of the eluate were collected and absolute radioactivity (DPM) was determined with a Searle Mark III liquid scintillation spectrometer, using quenched internal standards and a ^{133}Ba external standard. Positive identification of the ^{3}H-ara-CTP peak was made with the use of authentic nonradiolabeled ara-CTP (Sigma Chemical Co, St Louis) as an internal standard. This procedure permits effective separation of ara-CTP from dCTP.[28]

Determination of incorporation of ^{3}H-ara-C or ^{3}H-dThd into DNA. Following incubation, ^{3}H-ara-C incorporation into DNA of the blast cells was determined exactly as described previously for HL-60 cells.[30] ^{3}H-dThd incorporation into DNA also was determined by the same procedure except that cells were incubated with ^{3}H-dThd instead of ^{3}H-ara-C. Triplicate determinations were made for each experimental point except in those few patients in whom blast cell yield from the marrow was low, in which case determinations were made in duplicate. Radioactivity in zero-time background tubes was subtracted from the radioactivity of the appropriate experimental tubes. Radioactivity was converted to molar quantities using the known specific activity of the radiolabel.

Statistical methods. The data were analyzed by several statistical methods. When comparisons were made by *t* test, Student's *t* test was used if a comparison was made between two groups of individuals, and the paired *t* test was used when the observations being compared were from the same subject.

The statistical models were fitted using the GLM procedure in SAS (SAS Institute, Cary, NC)[31] using an IBM 4341 computer. Data were missing for one of the three ara-C concentrations for 5.9% of all observations. SAS treated missing values in such a way that any observation that had a missing value for any dependent or independent variable was excluded from the analysis.

RESULTS

Our research objectives were threefold: to study the relationship of dose of ara-C (10, 100 or 1,000 nmol/L) and the formation of potentially active metabolites of ara-C in blast cells from ANLL patients, to determine if the formation of such active metabolites of ara-C could be used to predict clinical response in individual patients, and to determine collectively if differences in ara-C metabolism exist among the three patient groups (CR, NR, and REL). To accomplish these objectives, using an in vitro culture system, we related the extracellular concentration of ara-C (dose) and the clinical response of a patient to three dependent variables which represent important events in ara-C metabolism: the amount of ara-C incorporated into DNA, the intracellular concentration of ara-CTP, and the ratio of these two measures, as a measure of efficiency of ara-C incorporation into DNA. The data and geometric means are shown in Figs 1 through 3. These plots are semilogarithmic. The means ± SE of the three dependent variables for each response group at each extracellular concentration of ara-C used are given in Table 1.

The data presented show a high degree of variability among patients in the amount of ara-C metabolite formed at any dose of ara-C (interpatient variability), contrasting with

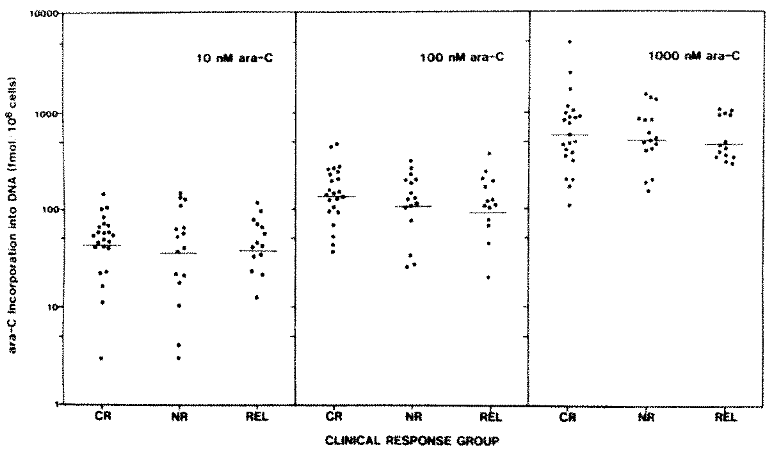

Fig 1. Cytosine arabinoside (ara-C) incorporation into DNA (fmol/10^8 cells) in response to exposure of patient marrow blast cells to 10, 100, or 1,000 nmol of ara-C. Each point represents the mean of duplicate or triplicate determinations obtained from a given patient's marrow blast cells at the dose of ara-C indicated. SDs were <15%. Horizontal bars indicate geometric means.

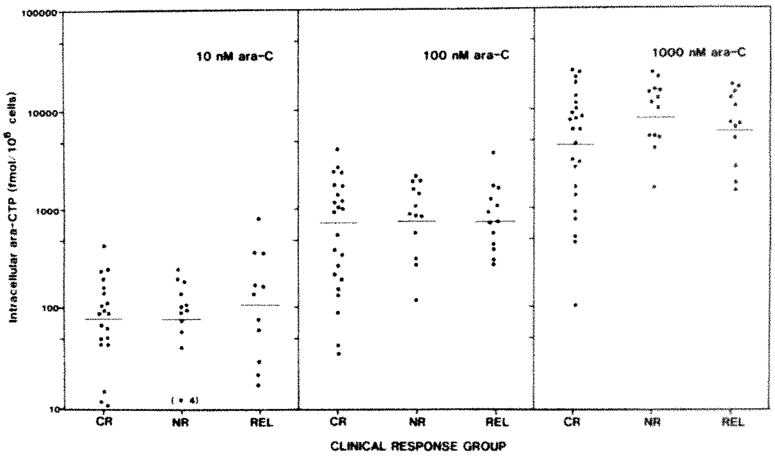

Fig 2. Intracellular cytosine arabinoside in triphosphate form (ara-CTP) formed (fmol/10^6 cells) in response to exposure of patient marrow blast cells to 10, 100, or 1,000 nmol/L of ara-C. Horizontal bars indicate geometric means.

the variability associated with making the measurements of ara-C metabolite in a single patient (experimental variability), which was small. For example, each data point in Fig 1 represents the mean of duplicate or triplicate experimental determinations for a single patient made at the corresponding dose of ara-C, with a SD for each point that was typically $< 15\%$. In addition, what is not apparent from Figs 1 through 3 is the fact that the rank placement of a particular patient's ara-C metabolite measurement at a given dose of ara-C tended to be constant at all other doses of ara-C. Thus, patients with relatively low incorporation at 10 nmol/L of ara-C also tended to have relatively low incorporation at 100 and 1,000 nmol/L as well. Thus, the variability seen in Figs 1 through 3 is really variability associated with differences among patients (interpatient variability) rather than experimental variability associated with the measurement of the ara-C metabolites of a given patient.

The data in Table 1 and Figs 1 through 3 reveal a clear positive relationship between ara-C dose and formation of ara-CTP or incorporation of ara-C into DNA in each clinical response group. Statistically significant increases were present at each increment of extracellular ara-C concentration by paired *t* test ($P < .0001$ in each case). Similarly, the efficiency of ara-C incorporation (ratio of incorporation/ara-CTP) decreased significantly with each increment in ara-C dose ($P < .0001$).

When the various clinical response groups (CR, NR, REL) are compared, Figs 1 through 3 reveal considerable overlap in ara-C metabolite formation among all clinical response groups. Indeed, no significant differences were found in ara-C incorporation, formation of intracellular ara-CTP, or efficiency of ara-C incorporation among the various clinical response groups by Student's *t* test ($P > .05$ in all cases). By inspection of these data, one can see readily that it would be impossible to predict, based on the ara-C metabolic variables of a given patient, the clinical response group in which that patient lies. Thus, at present, we are unable to fulfill our second goal, namely, the development of a test that will predict clinical response in an individual patient.

To ascertain collective trends among the patients studied, the data were evaluated by an analysis of covariance, a statistical method that uses the concepts of both analysis of variance and linear regression.[32] In computing the analysis of covariance, a natural logarithmic transformation was performed to stabilize the variance. For this reason, geometric means are provided in Figs 1 through 3. The results derived from this analysis agree substantially with the results obtained by *t* tests, as described above. Specifically, in relating ara-C dose with metabolite formed, we found that a 3.6-fold increase in ara-C incorporation into DNA and an 8.5-fold increase in intracellular ara-CTP can be expected

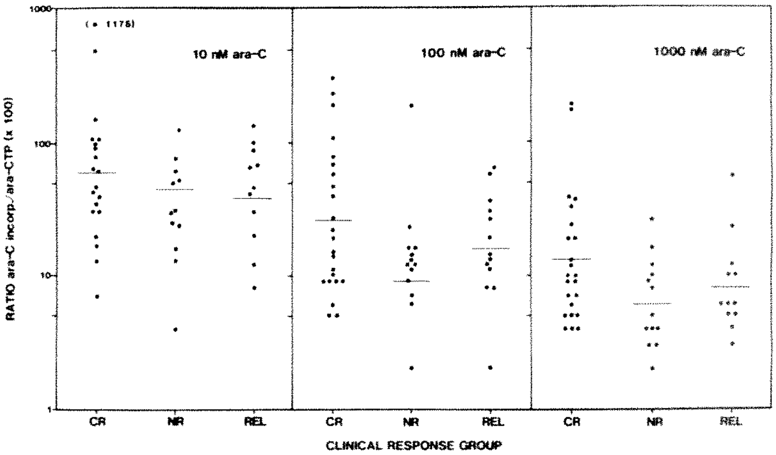

Fig 3. Efficiency of cytosine arabinoside (ara-C) incorporation into DNA [ratio of ara-C incorporation into DNA to intracellular ara-C in triphosphate form (ara-CTP)] expressed as a percentage. The ratio was obtained by dividing the incorporation of ara-C into DNA for a given patient at a given dose of ara-C (data shown in Fig 1) by the corresponding intracellular ara-CTP pool of that patient (data shown in Fig 2). Horizontal bars indicate geometric means.

Table 1. Arithmetic Means ± SE for ara-C Incorporation Into DNA, Formation of ara-CTP and Efficiency of ara-C Incorporation

Clinical Group	Extracellular Concentration of ara-C (nmol/L) 10	100	1,000
CR	54 ± 7 (23)*	165 ± 21 (23)	834 ± 202 (23)
NR	55 ± 12 (16)	131 ± 21 (16)	609 ± 105 (16)
REL	50 ± 8 (14)	126 ± 23 (14)	524 ± 77 (13)
Intracellular ara-CTP (fmol/10^6 cells)			
CR	116 ± 23 (20)	1,006 ± 207 (23)	7,931 ± 1,425 (23)
NR	105 ± 21 (13)	994 ± 188 (13)	10,139 ± 1,850 (13)
REL	179 ± 71 (11)	942 ± 236 (13)	7,781 ± 1,478 (12)
Efficiency of ara-C incorporation into DNA (ratio of incorporation to intracellular ara-CTP for each patient, expressed as %)			
CR	137 ± 59 (20)	57 ± 17 (23)	28 ± 11 (23)
NR	42 ± 10† (12)	25 ± 14 (13)	8 ± 2 (13)
REL	56 ± 13 (11)	23 ± 6 (12)	12 ± 4 (12)

Abbreviations as in Table 1.

*Numbers in parentheses refers to the number of patient marrows studied at that particular ara-C dose level.

†One patient was excluded whose ratio was 3,500%, which is >3 SD from the mean which includes this value.

for each 10-fold increment in the extracellular concentration of ara-C (*P* = .0001 in both cases) (Table 2). Because the rate of increase of ara-CTP was greater than that of ara-C incorporation, the efficiency of ara-C incorporation (ratio of incorporation to ara-CTP) decreased by a multiple of 0.42 (ie, a 58% decrease) with each tenfold increase in ara-C dose (Table 1).

In comparing the various clinical response groups, two separate analyses of covariance were done. The first analysis made the assumption that the patients studied were representative of the general population of patients with ANLL. The second analysis considered a special case in which the patients we studied constituted a unique population, not necessarily representative of the general population of ANLL patients. Small differences in the levels of the three dependent variables were noted (Table 3, adjusted mean values). The CR group, overall, incorporated more ara-C into DNA, had lower intracellular ara-CTP pools, and had a higher efficiency of incorporation of ara-C into DNA than did the other groups (NR and REL). However, in the first analysis, these differences were not statistically significant, consistent with the results of the *t* tests described above. In this case, the large amount of interpatient variability encountered prevented discernment of statistically significant differences. In contrast, the second analysis (which assumed that the patients were an entire population) found that the differences seen in the three dependent variables (Table 2) were large enough that they could not be due to experimental (or assay) variation, and thus they were statistically significant (*P* values, Table 2). However, conclusions drawn from this latter analysis must be limited to the group of patients studied and cannot be extended to the general population of ANLL patients.

Table 2. Analysis of Covariance: Relation of ara-C Metabolic Variable to ara-C Dose

ara-C Metabolic Variable	Slope*	*P* value
ara-C incorporation into DNA	3.6	.0001
ara-CTP	8.5	.0001
Ratio (incorporation/ara-C)	0.42	<.0001

*Slope equals multiple of change in cytosine arabinoside (ara-C) metabolic variable per tenfold increase in extracellular concentration of ara-C.

Six patients in the CR group were studied again at the time of relapse. These patients were not included in the REL group as described above. No statistically significant differences in the metabolism of ara-C were found among these patients at time of relapse as compared with their values before treatment.

Because ara-C is an S phase-specific agent, a possible confounding factor was the rate of DNA synthesis in each patient marrow sample. For each patient, we measured the uninhibited rate of DNA synthesis in the bone marrow cultures during the 22-hour period by measuring the rate of ^{3}H-dThd incorporation into DNA (means for each response group are shown in Table 4). No statistically significant differences in the rate of ^{3}H-dThd incorporation were found among the means of response groups by *t* test (Table 4). When the rate of DNA synthesis in each patient marrow sample was used as a covariable in the analysis of covariance, there was still a highly significant relationship between the ara-C metabolic variable (ara-CTP, incorporation or ratio) and the dose of ara-C.

DISCUSSION

These studies demonstrate a dose–response relationship for ara-C incorporation into DNA and formation of intracellular ara-CTP in blast cells from patients with ANLL. In response to doses of ara-C from 10 to 1,000 nmol/L, the increase in the rate of formation of ara-CTP is greater than that of ara-C incorporation into DNA. Thus, the efficiency of ara-C incorporation into DNA decreased significantly as the extracellular concentration of ara-C was increased. We attribute this effect in part to inhibition of DNA polymerase by the increased intracellular concentration of ara-CTP and in part to the fact that the extracellular concentrations of ara-C used were well below the K_m of deoxycytidine kinase for phosphorylation of ara-C (~20 μmol/L),[4] as evidenced by the linear and nearly direct proportional relationship we observed between extracellular ara-C concentration and intracellular pools of ara-CTP (Table 2). The studies of Riva and colleagues,[33] using high extracellular concentrations of ara-C (in excess of 20 μmol/L), found that the efficiency of ara-C incorporation increased with increasing ara-C dose, presumably due to saturation of the ara-C phosphorylating mechanism. We emphasize that, although we found that efficiency of incorporation decreased with increasing ara-C concentration, in no case did we observe an actual decrease in the absolute amount of ara-C incorporated into DNA by raising the extracellular concentration of ara-C.

The decrease in efficiency of ara-C incorporation with increasing dose of ara-C means that, relative to intracellular ara-CTP, a proportionately larger amount of ara-C is incor-

Table 3. Analysis of Covariance: Adjusted Mean Values

Ara-C Metabolic Variable	Response Group	Adjusted Mean	Units	*P* Value*
ara-C incorporated into DNA	CR	146.9		
	NR	124.6	f mol/10^6 cells	.0079
	REL	109.7		
Intracellular ara-CTP	CR	569.1		
	NR	823.0	f mol/10^6 cells	.0002
	REL	746.2		
Efficiency of ara-C incorporated into DNA	CR	27.4	Ratio: %, ie:	
	NR	15.6	(incorporation ara-CTP)	$<.0001$
	REL	18.4	× 100	

Covariance analysis adjusts the means of the dependent variables (ara-C incorporation, ara-CTP or ratio) by regression to estimates of what they would have been had they had a common independent variable (ara-C dose).[32] Thus, in comparing the various clinical response groups, covariance analysis provided single "adjusted mean" values for each response group. Adjusted mean values were obtained using the least squares option of the GLM procedure of SAS.

*Test of the hypothesis that the three means are equal if the patients studied are considered to be a unique population.

ara-C, cytosine arabinoside; CR, complete remission; NR, no response; REL, relapse.

porated into DNA at an extracellular concentration of 10 nmol/L, as compared with 1,000 nmol/L of ara-C. For example, by means of the slopes shown in Table 2, one can calculate that an increase in extracellular ara-C concentration from 10 to 1,000 nmol/L causes only an ~12-fold increase in ara-C incorporation into DNA, whereas intracellular ara-CTP increases ~70-fold. Ten to 100 nmol/L of ara-C approximates plasma concentrations obtained with "low-dose" ara-C therapy (eg, 10 to 20 mg/m^2 q 12 hours).[34] Perhaps the higher efficiency of incorporation of ara-C into DNA observed at lower doses of ara-C is involved with the efficacy of the "low-dose ara-C" regimen in producing complete remission in the treatment of ANLL, although the precise mechanism involved (differentiative *v* cytotoxic) must remain speculative at this time.

When our patients were considered as a unique population, those who entered CR (estimated clinically to be sensitive to ara-C) incorporated more ara-C into DNA with an equal or lower intracellular pool of ara-CTP than did those who were estimated clinically to be resistant to ara-C. Our finding of higher intracellular ara-CTP pools in clinically resistant patients agrees with the findings of Haanen and co-workers,[35] who observed increased ara-CTP formation in response to 1,100 nmol/L of ara-C in seven of eight ANLL patients at time of relapse as compared with their pretreatment (ie, at time of presentation) values. However, the significance of our findings cannot be extended to the general ANLL population at present since the interpatient variation encountered makes any comparison among response groups statistically nonsignificant when the patients are considered as a sample of the general ANLL population. Similarly, the interpatient variability encountered makes prediction of clinical outcome by such studies impossible in individual patients.

Table 4. In-Vitro Rate of ^{3}H-dThd Incorporation Into DNA (pmols/10^6 Cells ± SE)

Clinical Group	Incorporation
CR (N = 21)	10.9 ± 1.6
NR (N = 14)	17.2 ± 4.0
REL (N = 13)	9.2 ± 2.6

Abbreviations as in Table 1.

Marrow blast cells were incubated in vitro with ^{3}H-dThd in the absence of ara-C. There were no statistically significant differences in the means by Student's *t* test (CR *v* NR, $P > .5$; NR *v* REL, $0.1 > P > .05$; CR *v* REL, $p > .5$).

In cell lines selected for resistance to ara-C, common mechanisms of resistance are impaired phosphorylation of ara-C[3,12] or increased intracellular pools of dCTP.[13,14] Both mechansims should result in a markedly lower intracellular pool of ara-CTP as compared with nonresistant cells. Not only were we unable to demonstrate impaired production of ara-CTP in the resistant patients, but neither did we encounter any patient in this study whose blast cells were totally unable to generate intracellular ara-CTP. This includes patients whom one might consider to be "super-resistant" to ara-C: those in the NR group or those who relapsed while receiving the drug. This finding, combined with the trend for greater efficiency of ara-C incorporation into DNA in our clinically sensitive patients, implies that within our population of ANLL patients with varying clinical sensitivity to ara-C, differences may have existed at the level of DNA polymerase. We cannot, however, rule out enhanced intracellular dephosphorylation of ara-CTP or excision and repair of ara-C/DNA, since these mechanisms may only become apparent when the intracellular retention of ara-CTP or incorporated ara-C is determined, which was not done in our study.

The differences we observed among the clinical response groups were relatively small. For example, the adjusted mean for ara-C incorporation in the CR group was only ~25% greater than those of the NR or REL groups (Table 3). This small difference is not surprising when one considers that in ANLL, the frequency of drug-resistant cells necessary to cause the state of clinical resistance to therapy may be low (eg, <10% of the malignant cell population), since most patients in whom conventional ANLL induction therapy fails to produce CR achieve considerable marrow hypoplasia within 10 to 14 days following therapy. This implies that treatment with an ara-C regimen causes at least 1 log tumor kill (90% reduction of tumor), even in clinically resistant patients. If such is the case, the ara-C metabolites formed by the more populous nonresistant cells may mask the contribution of the resistant cells to the mean ara-C metabolite value obtained for the marrow blast cell sample. Indeed, the variations in ara-C metabolism of the nonresistant cells may have contributed markedly to the interpatient variability we observed in these studies. Thus, in detecting ara-C–resistant cells among a heterogeneous population of blast cells obtained from an ANLL patient, it may be necessary to use a sensitive method capable of analyzing multiple events in single cells. We are currently pursuing such an approach, using flow cytometry.

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Effects of Verapamil on In Vitro Intracellular Accumulation and Retention of Daunorubicin in Blast Cells From Patients With Acute Nonlymphocytic Leukemia

By Douglas D. Ross, Christopher C. Joneckis, and Charles A. Schiffer

The effects of verapamil on the intracellular pharmacokinetics of daunorubicin (DNR) in blast cells from the bone marrows of patients with acute nonlymphocytic leukemia (ANLL) were studied to determine whether verapamil was capable of enhancing intracellular accumulation and retention of DNR in ANLL, as has been observed in murine P388 cells resistant to DNR. Seventeen marrows from ANLL patients were studied, 13 of which were from patients who were considered to be clinically refractory to DNR. We took care to include such patients in this study since, in the P388 model, verapamil enhancement of DNR uptake is observed only in cells resistant to DNR. Intracellular accumulation of DNR was studied by exposing blast cells to DNR (1 μg/mL) ± verapamil (6.6 μmol/L) for up to 4 hours. Following a 2-hour exposure of cells to DNR ± verapamil, intracellular retention of DNR was studied by incubating the cells in DNR-free medium for 3 hours. Intracellular DNR/10^6 cells was quantified by fluorometry. In 12 of 15 patient marrows, verapamil failed to enhance intracellular accumulation of DNR. Three patient marrows had a very small increment in DNR uptake in response to verapamil (~14% greater than DNR alone) that was significant ($P < .05$) by paired *t* test. Intracellular retention of DNR ($t^1/_2$) and the area under the intracellular DNR *v* time curve (AUC) were studied in 17 patient marrow specimens. No significant alterations in these parameters were observed in response to verapamil. These data indicate either that verapamil did not substantially enhance DNR uptake or retention in blast cells obtained from ANLL patients who are clinically resistant to DNR, or that the frequency of DNR-resistant cells (ie, verapamil-responsive cells) among the blast cells obtained from these patients was too low to influence the population mean of intracellular DNR as measured in these studies.

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DAUNORUBICIN (DNR), an anthracycline antibiotic, is one of the most effective agents currently available for the treatment of acute nonlymphocytic leukemia (ANLL). When combined with the antimetabolite cytosine arabinoside (ara-C), high remission induction rates have been reported for previously untreated patients.[1,2] Unfortunately, most patients eventually relapse and die of leukemia despite continued therapy with DNR-containing regimens, suggesting the emergence or expansion of DNR-resistant leukemic cell populations in patients refractory to treatment.

Although a number of factors have been recognized that may play a role in the expression of cellular resistance to DNR,[3] the mechanisms of anthracycline resistance in human neoplasia are currently unknown. To date, a number of transplantable cells have been made resistant to DNR by exposure to anthracyclines in vivo or in vitro. These include cells of murine (eg, P388, Ehrlich ascites, L5178Y, MDAY-K2)[4-7] and human (HL-60)[8] origin. These resistant sublines seem to share a common mechanistic feature: namely, diminished ability to accumulate intracellular anthracycline as compared to wild-type (sensitive) cells, as a result of active outward transport of drug.[5,7,9-11] Many of these sublines display pleiotropic or multi-drug resistance (eg, to vincristine and many other antineoplastic drugs), and are associated with a cell surface marker (P-glycoprotein).[12]

Recently, calcium antagonists and calmodulin inhibitors have been found to enhance anthracycline intracellular accumulation and cytotoxicity in cell lines resistant to anthracyclines,[13-18] possibly by interfering or competing with the anthracycline for the active outward transport process. Similar effects of verapamil on vincristine uptake and cytotoxicity have been reported for P388/ADR cells,[14-18] a subline of the murine leukemia P388 which is resistant to ADR, DNR, vincristine, and other drugs through the multidrug resistance mechanism. A recent study[19] using blast cells obtained from patients with leukemia, however, reported the failure of verapamil to enhance vincristine uptake. Other investigators[20] failed to demonstrate the ability of verapamil (5 μmol/L) to enhance steady-state intracellular levels of DNR in blast cells from ANLL patients; however, only 5 of the 13 patients studied failed to achieve complete remission with a DNR-containing regimen. Because DNR is such an important element in the modern therapy of ANLL, we attempted to determine whether verapamil (6.6 μmol/L) can enhance in vitro intracellular accumulation and retention of DNR in blast cells obtained de novo from patients with ANLL.

MATERIALS AND METHODS

Patient selection. All patients studied had the diagnosis of ANLL established on the basis of microscopic examination of Wright's stained specimens of bone marrow, with French-American-British (FAB) subcategorization[21] established by examination of stained marrow specimens (α-napthyl esterase, chloroacetate esterase, terminal deoxynucleotidyl transferase, Sudan black, periodic acid-Schiff). FAB classifications and clinical status at the time of study are shown in Table 1. Of the marrows studied, four were from previously untreated patients who subsequently achieved complete remission (CR) with a DNR-containing induction regimen (clinically DNR-sensitive group). However, one of these patients (CB1) had a relatively short remission duration (4 months). These patients were treated with DNR (45 mg/m²/d, days 1, 2, and 3) and ara-C (200 mg/m²/day, days 1 through 7 by continuous intravenous (IV)

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Table 1. Patient Characteristics

Clinical Group	Clinical Response to DNR	Patient	FAB Classification	Status at Time of Study
CR	Sensitive	MG	M-4	Untreated
		WW	M-2	Untreated
		CBI 1	M-4	Untreated
		TJ	M-2	Untreated
NR	Resistant	AM	M-2	Untreated
		AG	M-2	Untreated
		WJ	M-1	Untreated
		GS	M-2	Untreated
		LE	M-2	Untreated
		SL	M-1	Previously treated
		CBu	M-5	Previously treated
		JI 1	M-2	Previously treated
		JI 2	M-2	Previously treated
		HH	M-2	Previously treated
REL (second)	Resistant	MP	M-2	Previously treated
(first)		PS	M-2	Previously treated
(first)		CBI 2	M-4	Previously treated

DNR, daunorubicin; CR, complete remission; NR, no response to therapy with a DNR-containing regimen; REL, patient in relapse.

infusion). Ten marrows were from patients who failed to achieve CR with a DNR-containing regimen and three were from patients in relapse (DNR-resistant group). Of the marrows from patients who failed to achieve CR, five were from patients prior to treatment, and five were from patients previously treated with a DNR-containing regimen as described above. It should be emphasized that all ten patients who did not achieve CR survived their induction attempt and therefore were fully evaluable for response to the DNR-containing regimen. Of the patients in relapse, patient MP was studied immediately prior to treatment of a second relapse, having had ten previous doses of DNR, 45 mg/m^2. This patient previously had experienced two relatively long remissions (duration 84 and 17 months) following treatment with the regimen of DNR and ara-C described above. Patient PS was studied immediately prior to therapy for a first relapse, having had five previous doses of DNR (45 mg/m^2) as part of initial induction therapy. Remission duration was 6 months. Patient CBI (remission duration 4 months) received three previous doses of DNR, 45 mg/m^2. None of the patients in relapse were treated subsequently with DNR.

Marrow collection and preparation. After informed consent, in accordance with our institutional guidelines, bone marrow (4.0 mL) was aspirated from the posterior iliac crest of patients using 0.1 mL of preservative-free heparin (1:1,000) as an anticoagulant. Freshly aspirated marrow was placed in a 15-mL sterile conical centrifuge tube (Costar Plastics, Cambridge, Mass), then diluted with an equal volume of RPMI medium 1640 (without serum) (GIBCO, Grand Island, Mich). Marrow cells were dispersed by gentle pipetting, and 5 mL of Ficoll-Hypaque ("LSM," Litton Bionetics, Inc, Kensington, Md) was layered under the marrow suspension. Mononuclear cells were collected at the Ficoll-Hypaque and medium interface after centrifugation (400 g for 40 min, 40 °C).[22] RBCs were lysed by exposure to 0.74% NH_4Cl, 4 °C,[23] following which the partially purified marrow mononuclear cells were washed once with ice-cold RPMI medium 1640 (without serum). Cells were resuspended in a volume of growth medium sufficient to achieve the desired final cell concentration (~0.8 to 1.2 × 10^6 cells per milliliter). The growth medium used was RPMI medium 1640 (GIBCO), containing 10% (vol/vol) heat-inactivated (56 °C for 30 minutes) fetal calf serum (FCS) (Sterile Systems, Logan, Utah). This procedure yields mononuclear cells of which 80% to 100% have the morphologic appearance of myeloid blast cells when viewed by light microscopy. Viability (trypan-blue dye exclusion) of the final blast cell preparation was usually > 90%, and was never < 80%. Median blast cell yield from the 17 patient samples was 1.9 × 10^8 cells, with a range of 0.3 to 5.0 × 10^8 cells. DNR uptake and retention studies commenced immediately at this point.

Blast cell culture and exposure to DNR with and without verapamil. Cells in growth medium were exposed to DNR (1 μg/mL) ± verapamil (6.6 μmol/L) at 37 °C, in an atmosphere containing 7.5% CO_2. Cell loss during incubation was minimal. For studies of DNR retention, cells in culture were exposed to DNR ± verapamil for 2 hours, after which the cells were placed on ice for 10 minutes, washed once in ice-cold phosphate-buffered saline (PBS),[24] then resuspended in warm (37 °C) growth medium with a cell density equal to that used during the 2-hour exposure to DNR. Incubation was then continued for 30 to 180 minutes. For cells exposed to DNR + verapamil, verapamil exposure was continued following removal of DNR. Cells were kept ice-cold during washing because influx and efflux of DNR in viable cells is zero when cells are washed under these conditions. Controls (4 °C) consisted of cultures incubated in the absence of DNR and then placed on ice for at least 10 minutes after which DNR ± verapamil was added. The purpose of the 4 °C controls was to control for cell surface or nonspecific binding of DNR and for diffusion of DNR into nonviable cells. The intracellular t½ of DNR was calculated by linear regression analysis; the area under the intracellular DNR *v* time curve (AUC) was calculated by the trapezoidal rule.[25]

P388 cell culture. The methods for culture of P388 cells have been described previously.[26] In brief, wild-type P388 cells (P388/S), obtained from Dr Randall Johnson (Smith, Kline and French Laboratories, Philadelphia), were maintained in liquid suspension culture at 37 °C, in 5% CO_2. The growth medium used was RPMI medium 1640 supplemented with newborn calf serum (10% vol/vol, Flow Laboratories, McLean, Va), L-glutamine (2 mmol/L), penicillin (50 IU/mL), streptomycin (50 μg/mL) and β-mercaptoethanol (0.01 μmol/L). Cells were placed in fresh medium every 5 days at an initial concentration of 2 × 10^5/mL. P388 cells resistant to DNR and ADR (P388/ADR), a gift from Dr Randall Johnson, were maintained in culture under conditions identical to those for P388/S cells described above.

Determination of cellular DNR content by fluorometry. The procedure is a modification of that previously described.[26] At the appropriate time after exposure of cells to DNR, cells were placed on ice for 10 minutes and were then washed once with 5 mL of ice-cold PBS. Following the PBS wash, cells were collected by sedimentation (400 g for 10 minutes at 4 °C), then lysed in 2 mL of 0.54 N H_2SO_4 in 75% (vol/vol) isopropanol. Particulate debris was removed by sedimentation (400 g for 10 minutes), and fluorescence of the supernatant was determined using a spectrofluorometer (Aminco-Bowman, American Instrument Co, Silver Spring, Md) with excitation wavelength of 470 nm and emission wavelength 585 nm. Fluorescence was converted to picomolar quantities of DNR by means of a standard curve, performed concomitantly. Fluorescence of 4 °C controls (background) was subtracted from each experimental point.

RESULTS

Uptake of DNR by leukemic blast cells. Using P388/S and P388/ADR cells in culture, we established experimental conditions that enabled demonstration of significant differences in intracellular pharmacokinetics between the two lines and demonstrated significant effects of verapamil on the intracellular pharmacokinetics of DNR in P388/ADR cells (Figs 1 and 2 show the results of a typical experiment). We

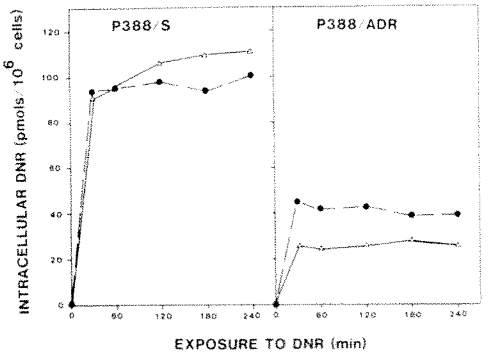

Fig 1. **Kinetics of intracellular accumulation of daunorubicin (DNR) in P388/S or P388/ADR cells. P388 cells in growth medium were incubated with DNR (1 μg/mL) with or without verapamil (6.6 μmol/L) at 37 °C, in 5% CO_2 for the time intervals indicated, after which they were washed once with ice-cold PBS (5 mL) and then lysed in 2 mL of 0.54 N H_2SO_4 in 75% (vol/vol) isopropanol. Particulate debris was removed by sedimentation. Fluorescence of the supernatant was determined (excitation 470 nm, emission 585 nm) using a standard curve to convert fluorescence units to amount of DNR. Data shown are the results of a typical experiment: Δ—Δ, DNR alone (1 μg/mL); ●—●, DNR + verapamil (6.6 μmol/L).**

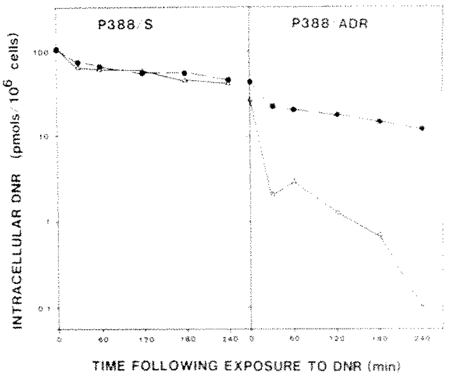

Fig 2. **Kinetics of intracellular retention of daunorubicin (DNR) for P388/S or P388/ADR cells following a 2-hour exposure to DNR (1 μg/mL) ± verapamil (6.6 μmol/L). Following the 2-hour exposure, the cells were washed once in ice-cold PBS, then resuspended in warm (37 °C) growth medium that did not contain DNR but, in the case of cells previously exposed to verapamil, contained verapamil (6.6 μmol/L). At the time interval following removal of DNR (as indicated), cells were removed for determination of DNR content. Data shown are the results of a typical experiment: Δ—Δ, control; ●—●, verapamil, 6.6 μmol/L.**

then applied these experimental conditions prospectively to blast cells obtained from patients with ANLL.

The effects of verapamil on the kinetics of intracellular DNR accumulation were studied in blast cells obtained from 15 marrow specimens (Table 2). One patient in this group (CBI) was studied on two occasions in different stages of his illness. Intracellular DNR content of cells exposed to DNR with or without verapamil for each patient at each time point was compared using the paired *t* test. Blast cells from only three patients (TJ, AG, and GS) displayed a significant increase in cellular DNR accumulation, as compared with control (DNR alone), when verapamil was added to the culture medium. Of these 3 patients, 2 failed to achieve CR with DNR (AG and GS), and 1 was clinically sensitive to DNR (TJ) (Table 1). The magnitude of verapamil-induced enhancement of DNR uptake in these patients was relatively small, however, with an average increment of 14.2% ± 8.2 (SD) above control for each time point. In the remainder of patient marrows studied, verapamil failed to alter blast cell accumulation of DNR significantly (Table 2). When the 15 marrow specimens were considered together, verapamil failed to cause significant differences in the kinetics or magnitude of intracellular DNR accumulation (Fig 3). In contrast, 6.6 μmol/L of verapamil caused an average incre-

Table 2. Effects of Verapamil on DNR Uptake by Human Leukemic Blast Cells

		Exposure to DNR (min)										
		No Verapamil						6.6 μmol/L of Verapamil				
Clinical Group	Patient	30	60	120	180	240	*P*	30	60	120	180	240
CR	MG	107	157	239	281	—	NS	130	157	212	192	—
	WW	117	179	327	323	408	NS	107	158	329	383	—
	CBI 1	109	184	245	258	391	NS	109	172	275	260	356
	TJ	75	108	218	249	268	.02	81	143	239	273	288
NR	AM	89	302	455	520	—	NS	72	345	459	584	—
	AG	65	75	128	132	142	.01	72	91	130	141	158
	WJ	147	160	241	284	—	NS	140	173	361	265	—
	GS	89	73	169	206	—	.05	103	91	199	228	—
	LE	87	176	257	301	312	NS	93	136	277	302	218
	SL	136	174	292	303	—	NS	125	153	300	426	—
	CBu	—	316	433	—	—	NS	—	316	432	—	—
	JI 2	—	382	494	628	—	NS	—	385	491	597	—
	HH	46	194	281	284	315	NS	66	105	307	303	313
REL	PS	318	482	655	893	—	NS	282	473	639	845	—
	CBI 2	513	575	689	741	—	NS	493	556	664	754	—

P value, paired *t* test. NS, not significant ($P > .05$); (—) not determined; other abbreviations as in Table 1.

Cells in culture were incubated with DNR (1 μg/mL) with and without verapamil (6.6 μmol/L) following which DNR uptake (pmol/10^6 cells) was determined, as described in the Materials and Methods section. Data shown are pmol of DNR/10^6 cells, and are the means of duplicate or triplicate determinations; SE < 10%.

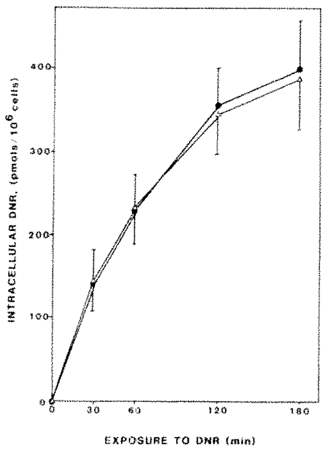

Fig 3. Kinetics of intracellular accumulation of daunorubicin (DNR) in blast cells from 15 marrow specimens obtained from patients with ANLL. The experimental procedures used in these studies were identical to those described in the legend to Fig 1. Data shown are the means ± SE (vertical bars) for all patient studies shown in Table 2. △—△, DNR alone (1 μg/mL); ●—●, DNR + verapamil (6.6 μmol/L).

ment of 64.4% ± 18.9% (SD) at each time point for the DNR-resistant cell line, P388/ADR (Fig 1). The data shown are the results of a typical experiment. Consistent with published reports,[13,17,18] verapamil failed to augment DNR uptake in the wild-type P388/S cells. Verapamil (6.6 μmol/L) failed to increase P388/ADR uptake of DNR to levels comparable to those observed in P388/S cells.

The magnitude and kinetics of intracellular DNR accumulation differ between human ANLL blast cells (Fig 3, Table 2) and murine P388 leukemia cells (Fig 1). P388 cells develop steady-state intracellular levels of DNR within 30 minutes of exposure to DNR, whereas intracellular DNR in human ANLL cells generally continues to increase over 120 to 180 minutes, with the blast cells of a few patients not reaching steady-state intracellular levels of DNR, even after 240 minutes of exposure to DNR (Table 2). In addition, the magnitude of intracellular accumulation of DNR at steady state is greater in cells from leukemic patients than in P388/S cells.

Retention of DNR by leukemic blast cells. The kinetics of elimination of intracellular DNR (Table 3) followed a biexponential pattern in the majority of the patients studied, with an initial, more rapid rate of decay observed within the first 30 to 60 minutes following exposure to DNR. However, in three patients (TJ, JI-1, PS), the decay was monoexponential. No differences caused by verapamil were noted in the initial decay phase (data not shown). The intracellular $t\frac{1}{2}$ (where decay was biexponential, $t\frac{1}{2}$ was based on the terminal intracellular elimination phase) and AUCs following removal of DNR were calculated for the 17 blast cell specimens (Table 4). Correlation coefficients (r) for the regression lines from which $t\frac{1}{2}$ values were calculated averaged .953 ± .057 (SD). Similarly, based on data shown in Fig 2, the AUC and $t\frac{1}{2}$ values were calculated for P388/S and P388/ADR cells, respectively. The results of a typical experiment are shown (Table 4, bottom).

As determined by paired t test, verapamil did not cause any significant alterations in intracellular $t\frac{1}{2}$ or AUC of DNR. In six patient studies (MG, WW, CBI-1, WJ, LE, SL)

Table 3. Effects of Verapamil on Intracellular DNR Retention by Human Leukemic Blast Cells

		Retention Time (min)									
		No Verapamil					6.6 μmol/L of Verapamil				
Clinical Group	Patient	0	30	60	120	180	0	30	60	120	180
CR	MG	239	—	103	116	—	212	—	98	69	—
	WW	327	304	272	229	181	329	282	292	220	198
	CBI 1	245	191	168	138	112	275	179	183	144	103
	TJ	218	—	—	167	149	239	220	204	172	172
NR	AM	455	371	331	281	271	459	401	346	333	291
	AG	128	93	82	60	55	130	88	87	67	58
	WJ	241	223	196	175	150	361	253	215	200	169
	GS	169	110	105	100	93	199	149	146	115	114
	LE	257	232	—	238	198	277	279	—	268	222
	SL	292	—	218	139	—	300	—	157	171	—
	CBu	433	354	309	261	189	432	330	312	234	205
	JI 1	134	106	120	81	68	136	107	105	81	73
	JI 2	494	—	366	330	323	491	—	324	289	291
	HH	281	—	203	173	159	307	—	206	196	166
REL	MP	354	—	248	198	158	362	—	216	165	130
	PS	655	542	502	425	331	639	498	482	502	421
	CBI 2	689	521	479	393	371	664	509	475	412	391

(—) not determined; other abbreviations as in Table 1.

Cells in culture were incubated with DNR (1 μg/mL) with and without verapamil (6.6 μmol/L) for 2 hours, following which DNR was removed by washing the cells in ice-cold PBS, then resuspending them in DNR-free medium, after which incubation was continued; samples were removed at the indicated time intervals for determination of intracellular DNR content. For cells previously exposed to DNR + verapamil, the resuspension medium contained 6.6 μmol/L of verapamil, with the exception of patients MG, WW, CBI 1, WJ, LE, and SL, in whom verapamil exposure was not continued following the removal of DNR. Intracellular DNR (pmol/10^6 cells) was determined as described in the Materials and Methods section. Data shown are pmol of DNR retained by 10^6 cells, and are the mean of duplicate or triplicate determinations; SE < 10%.

Table 4. Pharmacokinetics of Intracellular DNR Retention

Clinical Group	Patient	t½* Control	t½* + Verapamil	AUC† Control	AUC† + Verapamil
CR	MG	115.1	74.1	16,830	14,310
	WW	211.4	244.7	33,135	45,675
	CBI 1	169.9	145.6	28,605	29,460
	TJ	325.5	357.0	32,580	34,845
NR	AM	415.9	480.5	58,260	64,005
	AG	189.8	229.5	13,770	14,325
	WJ	276.1	283.4	34,125	39,750
	GS	645.7	338.9	19,530	24,405
	LE	487.4	611.8	41,685	47,655
	SL	112.1	147.9	26,010	23,550
	CBu	171.9	204.9	52,350	50,610
	JI 1	187.7	209.6	17,490	17,025
	JI 2	665.5	774.3	65,760	60,090
	HH	340.5	385.3	35,760	38,310
REL	MP	184.5	163.8	42,120	37,620
	PS	214.2	515.1	83,340	88,080
	CBI 2	294.9	384.6	81,540	82,740
	P388/ADR	90.1	238.7	696‡	4,440‡
	P388/S	300.8	327.5	13,815‡	14,573‡

*Minutes. Where decay curve was biexponential, terminal t½ values are given.

†pmol · min/10^6 cells.

‡AUC based on study which included a 240-minute time point.

exposure to verapamil was not continued following removal of DNR from the culture medium. The intracellular t½ or AUC of these patients did not differ as compared with those of patients in whom exposure to verapamil was continued following removal of DNR. Similarly, when studies of cells from these six patients or from the patients clinically sensitive to DNR were removed from this analysis, the result was the same: verapamil failed to prolong t½ or increase AUC. For the 11 patient studies in which exposure to verapamil was continued following removal of DNR, the combined kinetics of DNR retention ± verapamil is shown in Fig 4.

In contrast to the studies of DNR retention in human ANLL blast cells, verapamil increased the intracellular t½ of DNR from 90.1 to 238.7 minutes in P388/ADR cells (Table 4). Similarly, verapamil increased the AUC of DNR retention in these cells from 696 to 4,440 pmol · min/10^6 cells. Verapamil had minimal effects on the intracellular retention of DNR in P388/S cells. The t½ of P388/S cells was comparable to those of ANLL blast cells. The AUC, however, was lower in P388/S cells than in most ANLL blast cell populations studied, a reflection of the lower steady-state intracellular DNR content of P388 cells as compared with human ANLL cells.

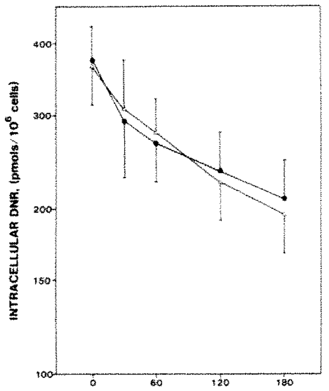

Fig 4. Kinetics of intracellular retention of daunorubicin (DNR) following a 2-hour exposure to DNR (1 µg/mL) ± verapamil (6.6 µmol/L) in blast cells from 11 marrow specimens obtained from patients with ANLL. The experimental procedures used in these studies were identical to those described in the legend to Fig 2. Data shown are the means for all patient studies shown in Table 3: Δ—Δ, control; ●—●, verapamil, 6.6 µmol/L.

DISCUSSION

Neoplastic cellular resistance to antileukemia medications is a major cause of failure of the therapy of ANLL today.[27] Although resistance to DNR probably arises through various mechanisms, the multiplicity and variety of cultured cell lines which display resistance to DNR and to other antineoplastic agents through an enhanced active outward transport (eg, multidrug resistance) mechanism[12] makes it reasonable to postulate that this mechanism will account for at least part of the resistance to DNR that develops in ANLL. Because cells resistant to chemotherapeutic agents by the multidrug mechanism display enhancement of intracellular uptake and retention of drug following exposure to verapamil, the demonstration of an in vitro enhancement of DNR uptake and retention by verapamil in blast cells from ANLL patients who are clinically resistant to DNR may provide indirect evidence for the existence of an active outward transport form of resistance in ANLL. The studies presented in this article cannot confirm this. We are dubious that the minimal enhancement of intracellular DNR accumulation noted in three patients is significant since no differences caused by verapamil were detected in intracellular retention of DNR in these patients. Moreover, one of these patients (TJ) was clinically sensitive to DNR. In addition, since the multidrug resistance mechanism commonly arises as a process of acquired resistance, not primary resistance, the patients in whom we would expect to see the most marked effect of verapamil would be those in the relapsed (REL) group and those in the no response (NR) group who were previously treated. Verapamil failed to influence intracellular pharmacokinetics of DNR in all of these patients.

Kessel and co-workers[20] studied the effects of 5 µmol/L of verapamil on the steady-state intracellular levels of DNR in marrow from 13 patients with ANLL, five of whom could be considered resistant to DNR, and failed to detect any alteration in intracellular DNR caused by verapamil. In this study, the 45-minute exposure of cells to DNR used was assumed to establish intracellular steady-state levels. This contrasts with our study, in which ANLL blast cells required times of up to 180 minutes to achieve intracellular steady-state concentrations (Fig 1).

P388 cells differed considerably from ANLL cells both in the magnitude and in the kinetics of intracellular DNR accumulation. If nonviable cells were present among the ANLL cells, it is possible the continued increase in intracellular DNR observed in ANLL was due to diffusion of DNR into the nonviable cells; this possibility was excluded since marrows with trypan blue viabilities <80% were not studied, and because the fluorescence of 4 °C controls (cells exposed to DNR at 4 °C) was subtracted from each experimental point. DNR will not enter viable cells at 4 °C, and thus fluorescence of the 4 °C controls represents the combined effects of DNR diffusion into nonviable cells and membrane and nonspecific binding of DNR.

Unlike cultured cells such as P388, in which drug-resistant sublines can be obtained with relative homogeneity, the blast cell populations obtained from patients with ANLL are heterogenous. One would expect variation among ANLL blast cell populations in the type of drug-resistant cells and variation within a population of ANLL blast cells in the frequency of drug-resistant cells. It is likely that the frequency of drug-resistant cells in ANLL necessary to cause the state of *clinical* resistance to DNR is quite low (eg, <10%), since most patients in whom therapy with a DNR-containing regimen fails to produce remission achieve considerable marrow hypoplasia within 10 to 14 days following therapy, implying at least 1 log cell kill. Thus, in blast cells obtained from patients clinically resistant to DNR, the contribution of the resistant cells to the population mean may be masked by that of the drug-sensitive cells in the population. Because the methods used measured population means, we feel that the present study, although a logical "first step" in the approach to the problem, does not answer definitively the question of whether verapamil enhances DNR uptake in ANLL, since the methodology used may fail to detect the contribution of DNR-resistant cells if they constitute a relatively small percentage of the total population. To answer this question, one must analyze the intracellular pharmacokinetics of DNR in individual cells within the population of ANLL blast cells, and we are currently pursuing such studies using flow cytometry.

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Natural History of Primary Infection With LAV in Multitransfused Patients

By the AIDS–Hemophilia French Study Group

In the course of a prospective study of asymptomatic, multitransfused subjects, seroconversion to human lymphadenopathy-associated virus (LAV/HTLV-III) occurred in 34 hemophilic and in two thalassemic patients. In subjects treated with procoagulant concentrates, primary infection, as evidenced by the development of antibodies to LAV, was a clinically silent event apart from moderate lymph node enlargement in 21% of cases. Concomitant immunologic disturbances mainly affected T lymphocyte subsets. This pattern contrasted with the major lymphadenopathy syndrome observed in the thalassemic patients who received washed erythrocytes from single donors positive for LAV antibodies. Four to 10 months after seroconversion, the incidence of lymphadenopathy reached 46% and the immunologic profile associated inverted T4$^+$/T8$^+$ lymphocyte ratio and markedly increased serum levels of IgG. In multitransfused hemophiliac patients, primary infection with LAV appears to provoke the following simplified sequence of events: decrease of T4$^+$ and increase of T8$^+$ cell counts preceding or concomitant with the occurrence of IgG LAV antibodies. Polyclonal elevation of IgG and lymph node enlargement occur weeks or months later.

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THE CLINICAL and biological picture of the lymphadenopathy syndrome (LAS), the acquired immunodeficiency syndrome (AIDS)—related complex (ARC), and full-blown AIDS is fairly well characterized.[1] In contrast, little is known about the early course of infection with the human lymphadenopathy-associated virus (LAV), also known as human T-lymphotropic retrovirus III (HTLV-III), aside from an Australian study in homosexuals[2] and a small number of posttransfusion or perinatally acquired cases.[3-6] Among primary infected patients, the factors responsible for the clinical diversity of LAV-related diseases remain to be determined.

In this report, a group of healthy and asymptomatic multitransfused patients consisting mainly of hemophiliac patients was prospectively studied. The clinical and biological changes which preceded, accompanied, and followed primary infection with LAV, as evidenced by a seroconversion, were analyzed. These findings were compared with those observed in groups of seropositive or seronegative subjects. The early stages of LAV infection transmitted by procoagulant concentrates were clinically nearly inapparent and differed from those occurring after other modes of contamination. However, the immunologic disorders were common to both.

MATERIALS AND METHODS

Population Studied

A survey of 425 symptom-free multitransfused patients was undertaken in late 1983 and early 1984 and results of this cross-section study have been reported.[7] This examination is subsequently referred to as initial checkup. Of this population, 146 subjects free of any clinical, hematologic, or immunologic abnormality were selected for a 1-year prospective study. Biological criteria for inclusion in the study were: platelet count >100,000/μL, total lymphocyte count >1,000/μL, ratio of T4$^+$/T8$^+$ lymphocyte subsets >1.0, serum level of IgG up to 1,600 mg/dL, delayed cutaneous hypersensitivity positive for two or more recall antigens, and serum alanine amino transferase less than three times upper normal level.

This selected population included 78 subjects with severe or moderate hemophilia A, 24 subjects with hemophilia A and an anti-VIII:C inhibitor, 26 subjects with hemophilia B, and 18 subjects with chronic anemia related to either thalassemia or sickle-cell disease (Table 1). This last subgroup met all selection criteria except serum level of IgG.

The prospective study was carried out over an average of 14 months per patient (range 11 to 17). Every 4 months (range 3 to 6) a clinical examination and hematologic and virologic checkup was carried out, including enumeration of platelets, lymphocytes, T4$^+$ and T8$^+$ cells, measurement of serum levels of IgG and IgM, and amino alanine transferase and skin tests with seven antigens. IgG antibodies to LAV (LAV Ab) were detected by immunoenzymatic assays.

The LAV Ab status was used as criteria to distribute patients into three groups: LAV Ab positive at onset of the study (45 subjects), LAV Ab negative throughout the study (65 subjects), and seroconverters (36 subjects). By convention, the date of the first LAV Ab sample in a previously LAV Ab-negative subject was considered as the time of seroconversion. In this report, we compare these groups, using the seronegative subjects as control. Results were evaluated on the basis of eight parameters recognized as indicative of LAV infection in the initial cross-section study[7] (Table 2). The amount of replacement therapy received by each patient was the sum of treatment received 1 year prior to entry and during the prospective study (range 22 to 29 months).

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Table 1. Population Under Study

Group	Seronegative No.	Seronegative Treatment (U/kg/m)	Seroconverter No.	Seroconverter Treatment (U/kg/m)	Seropositive No.	Seropositive Treatment (U/kg/m)
Hemophilia A*	22	53	22	98	19	177
Hemophilia A with† F VIII antibody	12	71	5	262	1	92
Hemophilia B‡	10	32	7	101	7	59
Chronic anemias§	16	1	2	1	—	—
Mean age (yr)	17.5		16.8		17.6	
(range)	(2–62)		(5–53)		(3–35)	

Treatment with: *factor VIII concentrates; †activated factor IX preparations; ‡regular factor IX concentrate, P.P.S.B.; §chronic anemia patients received monthly transfusions of packed erythrocytes, amounting to 200 mL/kg/yr.

Seropositive subjects. Forty-five hemophiliacs were seropositive for LAV Ab. Twenty-seven of these with complete records have been included in the prospective study. Nineteen patients with hemophilia A were treated with factor VIII; other patients were treated with factor IX concentrates.

Seronegative subjects. Seronegative patients with hemophilia A received factor VIII over a period of 24 months. During the last 12 months, the products were mostly of French origin. Patients with hemophilia A and antibody to VIII:C received either Autoplex (Travenol Laboratories, Glendale, Calif), FEIBA (Immuno AG, Vienna, Austria), or *PPSB* (Centre National de Transfusion Sanguine, Paris) over an average of 23.6 months. Patients with hemophilia B were treated over 25.6 months with *PPSB* concentrate prepared in France. The 16 patients with congenital anemia had received monthly transfusions of packed erythrocytes. Eight of them had undergone splenectomy in the past.

Table 2. Abnormalities of Index Parameters in Hemophiliacs

	Seronegative	Seroconverters Before SC*	Seroconverters At SC*	Seroconverters After SC*	Seropositive
No. of subjects	42	34	34	24	27
Lymphadenopathy	3	3	7	11†	14‡
Platelet count <100,000/μL	1	0	2	2	2
Lymphocyte count <1,000/μL	0	1	2	0	2
$T4^+$<600/μL§	11	6	9	4	8
$T8^+$>1,200/μL§	1	5	5	3	2
$T4^+/T8^+$<1.0	6	7	10	10	13
<0.8	4	6	7	8	8
Serum IgG > 1,600 mg/dL	7	7	12	15‖	16¶
Skin DH positive for fewer than two antigens	3	2	5	6	2

Differences between the groups of patients seropositive for at least 1 year and of those studied 5.3 months postseroconversion are not significant. Skin DH tests were not performed in any of the 27 seropositive patients.

*Seroconversion arbitrarily defined as the first seropositive sample. The intervals of sample collection before and after SC were 5.3 ± 1.8 and 5.3 ± 1.5 months, respectively.

†P = .016 (single-tailed exact test).

‡P = .000027 (single-tailed exact test).

§Cut-off values correspond to −1 SD from mean $T4^+$ count and to +2 SD from mean $T8^+$ count of the seronegative cohort.

‖$P < .05$ *v* preseroconversion.

¶$P < .05$ *v* seronegative.

Seroconverted subjects. The total amount of replacement therapy of these patients was calculated by adding the amount of product received during the year prior to the initial checkup and that which was received from the checkup date to the time of first seropositivity. Hemophilia A patients were treated with factor VIII preparations over a period of 21.8 months. Patients with anti-VIII:C antibodies received one or several of the three products mentioned above over a 22-month period. Hemophilia B patients were treated over 21 months. The therapeutic regime of the two thalassemic patients (aged 8 and 16 years) was identical to that of the 16 patients mentioned above; splenectomy had been performed in one.

Methods

Physical examinations were done blind with regard to the patient's anti-LAV status. Lymphadenopathy is defined as two extrainguinal areas with at least one lymph node >1 cm in diameter. Complete blood cell counts were performed on the model S counter (Coulter) and manually on a few occasions. T lymphocyte-membrane associated phenotypes were determined by indirect immunofluorescence using monoclonal antibodies (Ortho, Raritan, NJ) to helper/inducer ($OKT4^+$) and suppressor/cytotoxic ($OKT8^+$) T cell subsets. Absolute number of cells in each subset was calculated as the proportion of positive cells times the peripheral blood lymphocyte count. Normal values for subjects >10 years of age were 845 ± 110 and 533 ± 129/μL for $T4^+$ and $T8^+$ cells, respectively. In seronegative subjects, these values at two consecutive workups were 1,081 ± 473 and 698 ± 257, respectively. Delayed cutaneous hypersensitivity (DH) to seven antigens was tested with the Merieux Multitest (Lyon, France).[8] For each antigen, the DH was considered positive when the diameter of the induration was at least 2 mm at 48 hours. Because nearly all subjects had been immunized with BCG, diphtheria and tetanus anatoxins, DH was considered decreased when positive result was recorded for fewer than two recall antigens.

Virologic serum markers for hepatitis B, Epstein-Barr virus, and cytomegalovirus (HBV, EBV, CMV) were carried out by standard techniques. Coded samples were tested for IgG LAV Ab according to a slightly modified previously described enzyme-linked immunosorbent (ELISA) method.[9] Controls were run with plates coated with uninfected cells. For reactive sera, antibody titers were determined by comparing serial dilutions of test sample to a reference curve. Anti-LAV antibodies of seroconverters were characterized by an immunoblot technique using a standardized viral preparation[10] and, in some instances, by a radioimmunoprecipitation assay.[11,12]

Data were analyzed by the two-tailed *t* test for unpaired samples and, for comparing proportions, by chi-square statistics or Fisher's exact test.

RESULTS

Two of 18 seronegative subjects with chronic anemia seroconverted during the course of the prospective study. Details concerning these cases have already been reported, along with the presentation of another thalassemic patient who had his primary LAV infection a few weeks prior to the onset of the present study[4]; this subgroup will not be further analyzed. Among 78 hemophiliacs, who were seronegative at entry and monitored at regular intervals, 34 (44%) seroconverted during the 11 to 17 months of follow-up; this corresponds to a 37% annualized rate of seroconversion. There was no significant difference at the 5% level in the prevalence of seroconversion between hemophiliac patients treated with factor VIII or factor IX preparations. However, seroconverters treated with factor VIII or factor IX concentrates had received more products than their respective serologically negative controls ($P < .05$) (Table 1).

Irrespective of their type of therapy, seronegative hemophiliac patients had few abnormalities of index parameters. Two initially had an association of immunologic abnormalities: low $T4^+$ and elevated $T8^+$ counts, $T4^+/T8^+$ ratio <0.50 and serum IgG level above 1,600 mg/dL. This pattern was suggestive of primary infection with LAV, and additional tests were performed to seek evidence for viral transmission; the tests failed to detect LAV Ab. The other 42 subjects (Table 2) had dissociated abnormalities. Eleven patients had $T4^+$ counts <600/μL, causing a $T4^+/T8^+$ ratio <1.00 in six patients.

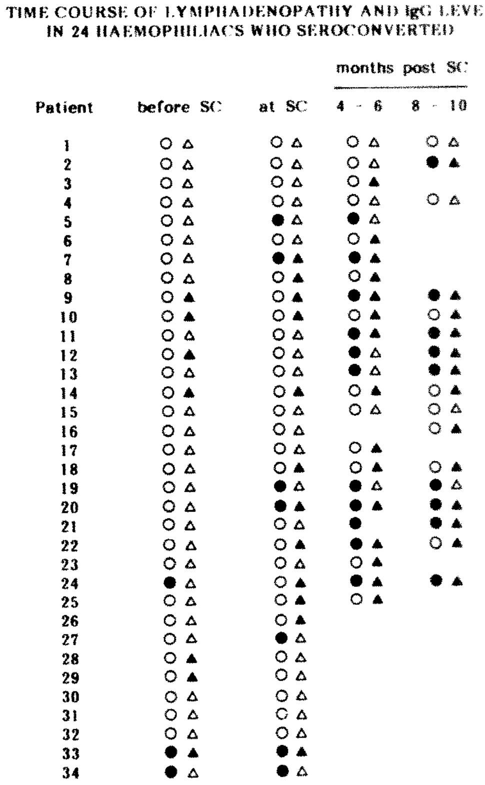
The occurrence of abnormalities of the eight index parameters in seroconverters was studied. For all patients, a checkup at 5.3 ± 1.8 months prior to seroconversion (in 11 subjects, it corresponded to the initial checkup) and at seroconversion were available; for 24 patients data, obtained at 5.3 ± 1.5 months postseroconversion were also analyzed (Table 2). Both the actual number of results outside normal ranges and differences from initial checkup indicate the presence of clinical and/or biological abnormalities in 19 patients several months prior to detection of anti-LAV IgG. These were essentially decreased $T4^+$ cell count, $T4^+/T8^+$ ratio, and reactivity to skin DH tests associated with elevation of $T8^+$ cell count and IgG level. When these subjects were compared with the group of patients who remained seronegative, it was difficult to evaluate whether the abnormalities were related to patients infected with LAV but still seronegative or to unspecific immunologic disturbances.

At seroconversion, lymphadenopathy was present in 21% of the patients, and the abnormalities seen before seroconversion were either similar or increased in number or magnitude. When differences from initial status were considered, ten subjects had significant changes in T lymphocyte subset counts related to either decreased $T4^+$ count or increased $T8^+$ count and (only once) to concomitant decrease of $T4^+$ and increase of $T8^+$ cells. The mean level of serum IgG was significantly higher than at entry (1,490 ± 460 *v* 1,250 ± 230 mg/dL, $P < .01$).

Examinations postseroconversion revealed a 46% incidence of lymphadenopathy ($P = .016$ *v* preseroconversion checkup), a normalization of $T8^+$ count and, in some cases, of $T4^+$ counts, although the $T4^+/T8^+$ ratio remained abnormal in ten subjects (Table 2). In contrast to this apparent improvement of T cell subset counts, serum IgG levels markedly increased in the 24 subjects who had postseroconversion checkups (mean 1,830 ± 460 mg/dL, $P < .001$ to baseline and $P < .005$ to level at seroconversion). Only two seroconverters remained normal for all index parameters throughout the observation period. Serum IgM levels were similar before and at seroconversion (195 ± 86 mg/dL and 194 ± 114, respectively). A slight decrease remaining below the level of significance was observed after seroconversion (166 ± 50 mg/dL).

Data obtained several months after seroconversion are remarkably similar to those observed in the 27 patients who were seropositive at the onset of the study and remained so over the 14 months of follow-up (Table 2). In this group, the incidence of lymphadenopathy, inverted $T4^+/T8^+$ ratio, and elevated IgG levels were 52%, 48%, and 59%, respectively.

The sequential occurrence of enlarged lymph nodes and elevated serum IgG levels in the seroconverters is shown in Fig 1. Overall, it appears that in a previously healthy patient with hemophilia, the occurrence of lymphadenopathy with concomitant decreased $T4^+$ cell count or increased $T8^+$ cell count, inverted $T4^+/T8^+$ ratio, and elevated serum IgG level are strongly suggestive of LAV infection. In only one patient, a flu-like syndrome was noticed in the weeks preceding



TIME COURSE OF LYMPHADENOPATHY AND IgG LEVEL IN 24 HAEMOPHILIACS WHO SEROCONVERTED

Patient	before SC	at SC	months post SC 4 - 6	months post SC 8 - 10
1	○ △	○ △	○ △	○ △
2	○ △	○ △	○ △	● ▲
3	○ △	○ △	○ ▲	
4	○ △	○ △	○ △	○ △
5	○ △	● △	● △	
6	○ △	○ △	○ ▲	
7	○ △	● ▲	● ▲	
8	○ △	○ ▲	○ ▲	
9	○ ▲	○ ▲	● ▲	● ▲
10	○ ▲	○ ▲	○ ▲	○ ▲
11	○ △	○ △	● ▲	● ▲
12	○ ▲	○ △	● △	● ▲
13	○ △	○ △	● △	● ▲
14	○ ▲	○ ▲	○ ▲	○ ▲
15	○ △	○ △	○ △	○ △
16	○ △	○ △		○ ▲
17	○ △	○ △	○ ▲	
18	○ △	○ ▲	○ ▲	○ ▲
19	○ △	● △	● △	● △
20	○ △	● ▲	● ▲	● ▲
21	○ △	○ △	●	● ▲
22	○ △	○ ▲	● ▲	○ ▲
23	○ △	○ △	○ ▲	
24	● △	○ ▲	● ▲	● ▲
25	○ △	○ ▲	○ ▲	
26	○ △	○ ▲		
27	○ △	● △		
28	○ ▲	○ △		
29	○ ▲	○ △		
30	○ △	○ △		
31	○ △	○ △		
32	○ △	○ △		
33	● ▲	● ▲		
34	● △	● △		

Fig 1. Checkups for lymph nodes (○) and IgG levels (△) in seroconverters. The presence of enlarged lymph nodes in at least two extrainguinal areas or IgG level above 1,600 mg/dL is indicated by closed symbols. SC denotes seroconversion. Intervals before and after SC are defined as in Table 2.

seroconversion. All other patients seroconverted asymptomatically aside from small-size lymphadenopathy.

IgG antibodies to LAV were analyzed by ELISA, immunoblot and, in some cases, radioimmunoprecipitation assay (RIPA) (Table 3). Variations in ELISA titers were observed from patient to patient. For a given subject, titers at seroconversion and in postseroconversion samples were stable, and the median titer was 1:800 in both series. However, in two patients, the titers increased significantly between the two samplings (1:100 to 1:1,600 and 1:200 to 1:1,600, respectively), which may indicate that the first blood specimen was drawn during an ongoing rise of IgG antibodies. ELISA-positive samples were checked by immunoblotting. All but one sample reacted with one or several LAV constitutive proteins. Analysis by RIPA, undertaken for selected samples, further revealed antibody to gp 110 in all 11 samples, including the ELISA-positive, Western blot-negative, second sample of patient 3. In ten postseroconversion sera, additional antibody specificities were detected, mainly against p34. No obvious pattern emerged from comparison of clinical and biological status of patients with antibody titers and/or specificities.

DISCUSSION

The population included in this prospective study was selected on the basis of normality of clinical, hematologic, and immunologic criteria. As expected, although it provided a standard background, the incidence of LAV Ab-positive subjects was lower (35%) than the general incidence observed in the cross-section study (47%),[7] due to the LAV Ab testing which took place several months after the prospective study was launched. The advantage was a relative homogeneity of the seronegative subjects, of whom 44% seroconverted.

Primary LAV infection caused by treatment of hemophiliac patients with factor VIII or IX preparations was a clinically silent event. The only clinical symptom was a moderate enlargement of peripheral lymph nodes. Data obtained from seronegative subjects indicated that, in this population in which 50% of the subjects were <18 years of age, some isolated lymphadenopathy may have other causes. This feature differs strikingly from reports concerning LAV seroconversion related to LAV Ab-positive washed RBCs, whole blood, and plasma, in which massive multifocal lymphadenopathy similar to LAS is described.[3-6,13] Among the 18 patients with congenital anemias enrolled in this study, two 8-year-old boys seroconverted. Both presented with large-size lymphadenopathy in all areas, similar to a patient already reported[4] who seroconverted before entry. In addition, although no other symptom was recorded, one of the two boys had a significant weight loss (4.5 kg) between the time of entry and seroconversion 9 months later. The acute mononucleosis-like syndrome described after accidental needle-stick transmission[14] and in a prospective study among Australian homosexuals[2] seems to correspond to the complete clinical picture of LAV primary infection. In our study, the progressive increase of the prevalence of lymphadenopathy before, during, and postseroconversion and their

Table 3. Follow-up of IgG LAV Ab in Seroconverters

		ELISA		Immunoblot†				RIPA‡			
Patient No.*		Screening	Titer	p18	p25	p34	p41	p18	p25	p34	gp110
3	SC	+	800	+	+						
	4 mos	+	800	–	–	–	–	–	–	–	+
4	SC	+	200		+			(+)	+	+	+
	4 mos	+	200		+	+					
11	SC	+	1,600		+			+	+	+	+
	4 mos	+	1,600		+	+	+				
12	SC	+	1,600		+						
	8 mos	+	800			+	+	(+)	+	–	+
13	SC	+	200		+						
	8 mos	+	1,600	+	+	+	+	+	+	+	+
14	SC	+	800		+			–	+	+	+
	8 mos	+	800		+			+	+	+	+
18	SC	+	1,600			+		+	+	+	+
	4 mos	+	1,600			+		(+)	(+)	+	+
22	SC	+	1,600		+	+	+	–	+	+	+
	8 mos	+	1,600		+	+					
24	SC	+	1,600	+	+	+	+	+	+	+	+
	8 mos		1,600		+	+					

ELISA, enzyme-linked immunosorbent assay; SC, seroconversion sample. Months are the number of months post-SC for the follow-up sample.

*Patients 3 through 14 were treated with factor VIII preparations; patients 18 through 24 received factor IX concentrates.

†Viral proteins p18, p25, p34, and p41 were identified as described in the Materials and Methods section.

‡RIPA denotes radio immunoprecipitation assay performed on 11 ELISA-positive samples; gp 110: membrane glycoprotein[12], (+) expresses a weak antigen-antibody reaction.

persistence over at least 5 months is strongly suggestive of inducement by LAV infection. Similar findings have previously been reported in hemophilic populations.[15-18]

Immunologic disturbances induced by replacement therapy with factor VIII concentrates in seronegative cohorts of hemophiliac patients have been described.[15-19] In this study, elevation of T suppressor cells and sharp rise in IgG serum levels appeared related to the LAV infection (Table 2). This observation made in the seroconverters was confirmed by our findings in the seropositive group of patients. Persistent lymphadenopathy and elevation of T8$^+$ cells and serum IgG levels may thus be considered as reliable markers of LAV infection, particularly when they are associated (Fig 1). However, any of these three parameters can be separately altered over time in a given subject. In contrast, low T4$^+$ cell counts were randomly distributed among seronegative and seropositive groups. A moderate decrease in T4$^+$ may therefore reflect in part the effects of replacement therapy itself. T helper cells are known to be the privileged target of LAV; our data, however, did not single out a specific defect of this subset, which seems more prone to abnormalities related to other causes, such as EBV, HBV, or CMV chronic infections. Repeated evaluation of delayed hypersensitivity for seven antigens by the Multitest appeared to be a useful tool for evaluation of immune reactivity. Although this repetition has been reported to modify the extent of response, the number of responsive antigens was not affected.[8]

From the collected data, a simplified sequence of event appears to be: decrease in T4$^+$ and increase of T8 counts preceding or concomitant with occurrence of IgG LAV Ab, followed by polyclonal elevation of circulating IgG and lymph node enlargement (Fig 2).

Our data confirm previous findings demonstrating that seroconversion is not a reflection of an immunization by incomplete noninfective viral antigens but rather is a replicating virus hosted by T lymphocytes.[20] It is possible that the mild primary infection syndrome observed in hemophiliac patients is caused by a small number of infective particles deriving from few donors whose plasma is highly diluted in a large pool. A possible attenuation of viral pathogenicity related to purification procedures and freeze-drying may also play a role. Whether such a small infective dose carries a better long-term prognosis remains to be evaluated.

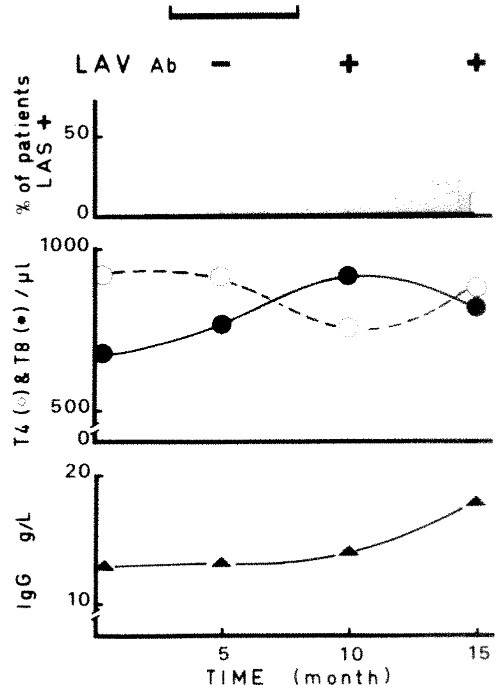


Fig 2. Schematic diagram picturing the sequential values of T lymphocyte subset counts, serum IgG levels (patients <10 years of age omitted), and prevalence of lymphadenopathy (LAS) in seroconverting hemophiliac patients. Bar (top) corresponds to the likely period of LAV contamination. Means of data from 24 patients >10 years of age were used to draw this diagram.

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Covalent Crosslinking of von Willebrand Factor to Fibrin

By Masao Hada,* Marek Kaminski, Paula Bockenstedt, and Jan McDonagh

Factor XIIIa crosslinks a limited number of substrates via $\epsilon(\gamma$-glutamyl)-lysyl bond formation. It crosslinks fibrin to itself, α_2-plasmin inhibitor and fibronectin to fibrin, and fibronectin to collagen. Results presented here show that plasma von Willebrand factor (vWF) is a substrate for factor XIIIa and can be crosslinked to fibrin during gel formation. vWF-fibrin crosslinking was studied in purified systems and in plasma with ^{125}I-vWF and ^{131}I-fibrinogen. vWF incorporation into fibrin increased with time or increasing factor XIIIa. After electrophoresis of dissolved clots, distribution of ^{125}I and ^{131}I was measured and showed that vWF was crosslinked to the α chain of fibrin and entered the high-mol-wt α polymer. vWF-fibrin crosslinking decreased the initial rate of α polymer formation. Crosslinking of vWF polymer to itself could not be demonstrated under physiologic conditions but occurred if vWF was reduced first. Factor XIIIa catalyzed incorporation of putrescine into both monomeric and polymeric vWF. Altogether, these studies indicate that factor XIIIa can readily form covalent bonds between glutamine in vWF and lysine in fibrin α chains. This reaction occurs readily in vitro when plasma clotting is slow and may occur in vivo under similar conditions.

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VON WILLEBRAND factor (vWF) is a large, multimeric plasma glycoprotein component of the factor VIII complex. vWF is important in the initial adhesion of platelets to the vascular subendothelium, and the molecular mechanisms by which it facilitates platelet adhesion have been partly elucidated. vWF has been shown to bind both to the platelet receptors, glycoprotein Ib[1,2] and glycoprotein IIb-IIIa,[3,4] and to collagenous[5,6] and noncollagenous components of the subendothelium,[7] thus forming a bridge between activated platelets and damaged endothelium.

Fibrinogen, in particular, and also fibronectin, bind to the platelet glycoprotein IIb–IIIa receptor, which raises the possibility of specific vWF interaction with these proteins that also facilitate platelet adhesion and aggregation.[8-10] Purification of each of these proteins from plasma is hampered by contamination with the other two. Fibrinogen is necessary for the cryoprecipitation of vWF, and both fibrinogen and fibronectin are required for heparin-induced precipitation of vWF.[11,12] vWF also precipitates in the presence of fibrin–fibronectin complexes.[11] The conditions under which vWF forms aggregates with fibrin(ogen)–fibronectin are nonphysiologic, and it is difficult to determine if such aggregate formation has physiological relevance. However, it is possible that similar interactions between fibrin and vWF may occur at sites of vessel wall injury where glycosaminoglycans on damaged endothelial surface or in the matrix may be available.

It has previously been reported that serum from hemophiliacs with long clotting times contains less vWF than do the corresponding plasma samples.[13,14] This was only observed in in vitro clotting systems when the clotting time was prolonged. However, it was also observed in normal samples when the clotting time was long.[14] From this it was hypothesized that a time-dependent interaction between vWF and fibrin could occur during clotting under appropriate conditions. Further studies with factor XIII-deficient or factor XIII-supplemented plasma indicated that this interaction might involve covalent crosslinking of vWF to fibrin.[14]

In the present study, the interaction of vWF with fibrin in purified test systems and in plasma clotting systems has been characterized. Evidence is presented that vWF is a substrate for the transglutaminase reaction catalyzed by factor XIIIa. Monomeric vWF can covalently crosslink to itself, but the polymeric forms do not. However, under appropriate conditions vWF polymers can be covalently crosslinked to the α chains of fibrin during the process of fibrin gel formation. This reaction can occur in plasma and requires only that the gelation process be long.

MATERIALS AND METHODS

von Willebrand factor purification and radiolabeling. vWF was purified from human plasma (obtained from the American Red Cross Blood Services, Northeast Region) by a modification of the procedure of Kao and colleagues,[15] as previously described.[16] Protein concentrations were determined by the method of Lowry and co-workers[17] and by electroimmunoassay. Purity was assessed by sodium dodecyl sulfate (SDS) gel electrophoresis under reducing conditions.[18] vWF activity was assayed as ristocetin-dependent platelet agglutinating activity (vWF:Ag).[16] Purified material was labeled with ^{125}I by the Iodobead procedure.[19] One Iodobead was preincubated with 0.5 to 1.0 mCi Na^{125}I and 100 μL of 50 mmol/L of Tris-0.15 mol/L of NaCl–0.02% azide, pH 7.2, for 10 minutes at room temperature; 500 μg of purified vWF was then added. After 10 minutes, iodination was stopped by transfer of the vWF solution to a column of Sephadex G-25 (0.7 × 10 cm) preequilibrated with Tris-saline buffer containing 1 mg/mL of bovine albumin. Labeled vWF was eluted in the same buffer. With this procedure, the average specific radioactivity of ^{125}I-vWF was 0.20 to 0.25 μCi/μg of protein. The vWF concentration agreement between the two methods, electroimmunoassay and vWF:Ag was good ($N = 16$, $r^2 = 0.993$), indicating that radiolabeling did not have a deleterious effect on functional properties of vWF.

Fibrinogen preparations. Fibrinogen was purified from human plasma by $(NH_4)_2SO_4$ precipitation.[20] The product was 93% clottable and showed the three expected bands (Aα,Bβ,γ) on reduced SDS gels. For some experiments, fibrinogen was treated with *p*-hydroxymercuribenzoate to inactivate trace amounts of factor XIII in the preparation.[21] After this treatment, the clottability was 91%. vWF contamination in the fibrinogen preparations was determined by electroimmunoassay and ranged from 15 to 25 μg/mg of fibrinogen.

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In some experiments, this was used as a source of vWF. In experiments using radiolabeled fibrinogen, the fibrinogen preparation was freed of vWF by affinity chromatography on fibrin monomer Sepharose before labeling.[22] vWF was eluted by 50 mmol/L of Tris-H_3-PO_4–0.1 mol/L of NaCl-25 mmol/L EACA, pH 7.6. Fibrinogen was eluted by Tris-H_3PO_4-EACA buffer containing 1 mol/L of NaCl, pH 4.1. The thrombin inhibitor, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (Calbiochem-Behring, La Jolla, Calif), was added to the sample and to the elution buffers. After elution, fibrinogen was dialyzed *v* Tris-saline, pH 7.4, and concentrated by precipitation with polyethylene glycol. This fibrinogen was labeled with ^{131}I by the solid-phase lactoperoxidase-glucose-oxidase procedure using Enzymobeads (Bio-Rad, Richmond, Calif).[22] The specific radioactivity was 0.025 to 0.035 $\mu Ci/\mu g$ of protein.

Factor XIII. Factor XIII was purified from human plasma or placental concentrate as previously described.[23] Placental concentrate (Fibrogammin) was kindly provided by Behringwerke A.G.; it contained the intracellular form of factor XIII (equivalent to platelet factor XIII, A_2) and primarily albumin. Because in these studies the same results were obtained with purified factor XIII and with the concentrate, the concentrate was routinely used without further purification. In the concentrate, 1 U is equal to the amount of factor XIII in 1 mL of normal plasma. Factor XIII activity was assayed by the ^{14}C-putrescine incorporation assay with activation of the zymogen by thrombin and $CaCl_2$.[24] In experiments in which factor XIIIa was added directly to the test system, the zymogen was treated with 1 U/mL of thrombin and 10 mmol/L of $CaCl_2$ for 30 minutes at room temperature. After cleavage of the factor XIII A chain, thombin was inhibited by addition of PPACK or hirudin, which did not interfere with factor XIII enzyme activity.

Putrescine incorporation into vWF. The substrate suitability of vWF for the crosslinking reaction catalyzed by factor XIIIa was determined by measuring the covalent incorporation of ^3H-putrescine or ^{14}C-putrescine (New England Nuclear, Boston) into purified vWF in the presence of factor XIIIa. The following experimental conditions were used: 0.99 μmol/L of vWF, 50 mmol/L of putrescine, 16.7 mmol/L of dithiothreitol, 6.7 mmol/L of $CaCl_2$, and 21 U/mL of thrombin. Factor XIII was varied from 0 to 5 U/mL. In some experiments hirudin (83 U/mL) was added after factor XIII activation and before addition of vWF to the test system. Incorporation of putrescine into vWF was allowed to proceed at room temperature for 2 minutes to 18 hours. The reaction was terminated by spotting samples on filter paper and immediately immersing them in cold 10% trichloroacetic acid (TCA) for 20 minutes. Filter papers were transferred to 5% TCA and then washed three times each in ethanol-acetone (1:1) and 100% acetone. Samples were air dried and counted in a scintillation counter. The recovery of vWF by this technique was 97.8% to 100%.

Covalent incorporation of putrescine into vWF was also analyzed by SDS gel electrophoresis. Samples were similarly prepared (50 mmol/L of ^3H-putrescine, 0.99 μmol/L of vWF, 6.7 mmol/L of $CaCl_2$, 21 U/mL of thrombin, 0 to 5 U/mL factor XIIIa); incorporation was allowed to proceed for 18 hours at 4 °C; it was terminated by addition of SDS sample buffer containing dithiothreitol, followed by incubation at 100°C for 5 minutes. SDS gel electrophoresis was carried out on 4% cylindrical gels under reducing conditions.[25] Gels were sliced in 1-mm sections and dissolved in 30% H_2O_2 overnight at 56 °C, and total radioactivity was measured.

Covalent crosslinking of vWF to fibrin in a purified system. For these experiments factor XIII (0 to 50 U/mL) was preactivated with thrombin (5 U/mL) and $CaCl_2$ (10 to 100 mmol/L) for 30 minutes at 37 °C. In the second step, the activation mixture was diluted fivefold to tenfold with ^{131}I-fibrinogen and ^{125}I-vWF, and clotting was allowed to proceed for 2 minutes to 18 hours. Fibrinogen, which had no factor XIII activity, was 1.4 to 1.9 mg/mL; vWF was 48 to 56 μg/mL for the test samples and 0 in the controls. Test systems also contained 10 μL ^{131}I-fibrinogen (78,300 cpm/μg) and 10 μL ^{125}I-vWF (10,700 cpm/μg). At various time intervals, clots were separated, compressed to remove the fluid, and washed with Tris-saline containing 50 mmol/L of EDTA. Total radioactivity in the clots and supernatants was measured. Supernatants were also assayed for vWF antigen by electroimmunoassay. Washed clots were dissolved in urea–SDS–DTT sample buffer and analyzed by SDS gel.[25] After electrophoresis, the gels were sliced in 1-mm sections and counted in a γ-spectrometer.

Covalent crosslinking of vWF to fibrin in a plasma system. Normal plasma (fibrinogen, 2.4 to 2.6 mg/mL; vWF antigen 10 to 15 μg/mL) and plasma from a patient with severe hemophilia A (fibrinogen, 2.6 mg/mL; vWF antigen, 20 μg/mL) were allowed to clot by addition of $CaCl_2$ (10 mmol/L). In some experiments, clotting times were adjusted by adding hirudin to normal plasma or thrombin to factor VIII-deficient plasma. In some experiments, trace amounts of ^{131}I-fibrinogen and ^{125}I-vWF were added to the plasmas before clotting. Clots were allowed to form for 3 hours at 37 °C and overnight at 4 °C. Clots and sera were then analyzed for vWF and fibrin as in the test system, using purified reagents.

Electroimmunoassay. vWF antigen was quantitated by Laurell immunoelectrophoresis.[26] Electrophoresis was performed in 0.9% agarose gels, containing 0.65% monospecific rabbit antiserum to human vWF (Calbiochem-Behring, Marburg, FRG), in Tris-barbital buffer (pH 8.6, ionic strength 0.03) at constant current (1 mA/cm) at 4 °C for 18 hours. Gels were stained with Coomassie blue and destained with glacial acetic acid–methanol–water. There were no changes in vWF concentration when factor XIII was added to the mixture.

RESULTS

Measurement of the covalent incorporation of a lysine analogue into a protein as a function of factor XIII activity is a straightforward way to determine if the protein contains glutamine(s) that may be suitable substrate(s) for transglutaminase catalyzed crosslinking. Figure 1 shows the results of such an experiment for purified, monomeric vWF. In the presence of factor XIIIa, vWF covalently incorporated putrescine in the expected time-dependent and concentration-dependent manner. At 1 hour, 0.97 μmole of putrescine

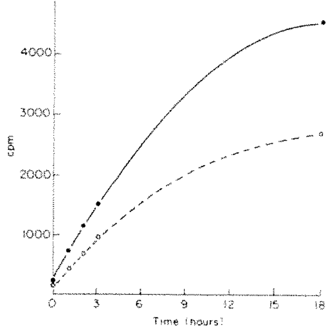

Fig 1. Covalent incorporation of ^3H-putrescine into von Willebrand factor by factor XIIIa. Factor XIII was activated with thrombin and $CaCl_2$ plus DTT, and then hirudin was added. vWF (0.495 mg/mL) was added to factor XIIIa and ^3H-putrescine. At various times, aliquots were placed on filter paper, washed with TCA, ethanol:acetone, and acetone, and counted; (---) 1 U/mL of factor XIII; (——) 5 U/mL of factor XIII.

was covalently bound per μmole of vWF monomer when vWF was incubated with 1 U/mL of factor XIIIa. Increasing the factor XIIIa to 5 U/mL increased the incorporation at 1 hour to 1.55 μmoles of putrescine incorporated per micromole of vWF monomer. These results clearly indicate that under certain experimental conditions factor XIIIa readily incorporates putrescine into glutamine residues of monomeric vWF. Further studies to define the conditions for this reaction showed that both thrombin and reducing agent enhanced the rate of incorporation, indicating that thrombin proteolysis and reduction exposed additional glutamines that could participate in crosslinking. After 18 hours of incubation, there was ~20% enhancement in the total putrescine incorporation in the presence of thrombin.

Further investigation of vWF crosslinking showed that when purified vWF was fully reduced and incubated with factor XIIIa, not only did monomeric vWF incorporate putrescine, but it also covalently crosslinked to itself (Fig 2A). In contrast, incubation of vWF polymer with factor XIIIa did not result in vWF crosslinking to itself (Fig 2B). As shown in Fig 2A, vWF was fully reduced by addition of dithiothreitol to the incubation mixture. After 18 hours, samples were subjected to SDS gel electrophoresis in reducing buffer; gels were then sliced into 1-mm sections and radioactivity was measured. The results show radioactive bands migrating in positions corresponding to vWF monomer, dimer, and trimer, as well as higher polymer formation. This indicates that vWF monomer has both glutamine and lysine groups that can participate in the crosslinking reaction. However, incubation of purified vWF polymer with factor XIIIa resulted in incorporation of putrescine but not in covalent crosslinking of vWF with itself, indicating that in the polymeric state only glutamines, not lysines, were available for crosslinking.

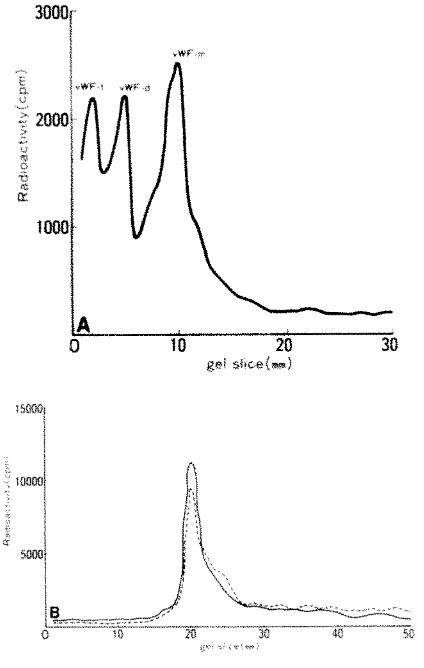

Fig 2. Crosslinking of von Willebrand factor by factor XIIIa. (A) Monomeric vWF was incubated with factor XIIIa and ^{3}H-putrescine as in Fig 1 with 1 U/mL of factor XIII. The crosslinking reaction was terminated by mixing samples with sodium dodecyl sulfate (SDS) reduced gel electrophoresis buffer, and electrophoresis was carried out in cylindrical gels. Gels were cut in 1-mm transverse sections, dissolved in H_2O_2, and counted. (B) Polymeric ^{125}I-vWF plus $CaCl_2$ was incubated in the presence (---) or absence (——) of factor XIIIa for 4 hours and then analyzed by reduced SDS gel electrophoresis. Gels were cut in 1-mm sections and counted.

Covalent crosslinking of vWF to fibrin was investigated in test systems containing purified vWF (trace-labeled with ^{125}I) with purified fibrinogen (trace-labeled with ^{131}I). Factor XIII was activated with thrombin and $CaCl_2$, and this mixture was added to the fibrinogen–vWF mixture to initiate clotting and crosslinking. All the clotting reactions were carried out in the absence of reducing agents, that is, with intact fibrinogen and vWF polymer. In some studies, after termination of the reaction, the reaction products were reduced and analyzed on reduced SDS gels. Figure 3A shows the covalent crosslinking of vWF to fibrin at various factor XIIIa concentrations. In this experiment, the molar ratio of fibrinogen to vWF monomeric unit was 30:1; at the highest

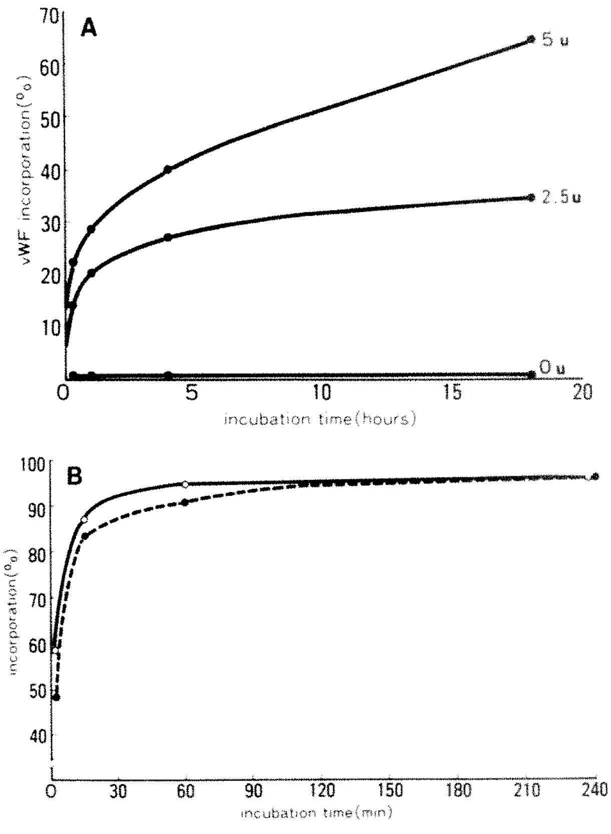

Fig 3. Incorporation of vWF into fibrin gels. (A) Factor XIIIa dependence. Fibrinogen (factor XIII-free) plus vWF (molar ratio 30:1) was clotted with thrombin and $CaCl_2$ and varying concentrations of factor XIII. Factor XIII concentration is shown at the right of each curve. At various times, clots were separated from fluid, washed in EDTA, and vWF was measured by electroimmunoassay. (B) Effect of vWF on fibrin incorporation. Samples were similarly prepared except that trace ^{131}I-fibrinogen was added. Fibrin in the washed clots was measured by radioactivity in the presence (---) or absence (——) of vWF.

factor XIIIa concentration tested, 80% of the vWF was covalently bound to fibrin. Analysis of the material in fibrin–vWF clots showed that the presence of vWF had a small effect of the rate of the fibrin gel formation (Fig 3B). At 2 minutes, 58% of the fibrinogen in the test system was in the clot in the sample without vWF, and 48% was in the clot in the sample with vWF. At 15 minutes, the values were nearly the same (87% without vWF and 85% with vWF); by 2 hours, there was no difference in the fibrin content of the two clotting systems.

Composition of the fibrin–vWF gels was analyzed by reduced SDS gel electrophoresis. Figure 4 shows the radioactivity profile of vWF and the fibrin polypeptide chains for fibrin formed in the presence or absence of vWF. At the earliest time point (2 minutes) fibrin γ chain content was decreased and γ-γ dimers were prominent. Fibrin α chains were also somewhat decreased, and small oligomers of α chains were visible, as well as some high-mol-wt α polymer. At this point, a small amount of vWF-α chain dimers and some larger components were seen, but there was no vWF in the α polymer fraction. In this assay, monomeric vWF, derived from vWF polypeptides that had been disulfide-bonded to other polypeptide chains that had become crosslinked to fibrin, was also observed. A principal effect of vWF appeared to be to decrease the rate of high-mol-wt α polymer formation. Similar results were observed at 15 minutes, at which time some vWF had appeared in the α polymer fraction and the sample without vWF still contained more α polymer than the sample with vWF. By 4 hours, most of the vWF had become crosslinked into the α polymer fraction, with residual vWF monomers and vWF-α chain dimers remaining.

Competition between vWF and fibronectin for crosslinking to α chains was investigated in a purified test system in which 94% of the fibronectin and 88% of the vWF was crosslinked when each was added separately to the clotting mixture. When both were present in the test system, identical amounts of both vWF and fibronectin were crosslinked to fibrin, indicating that there was no competition for α chain sites.

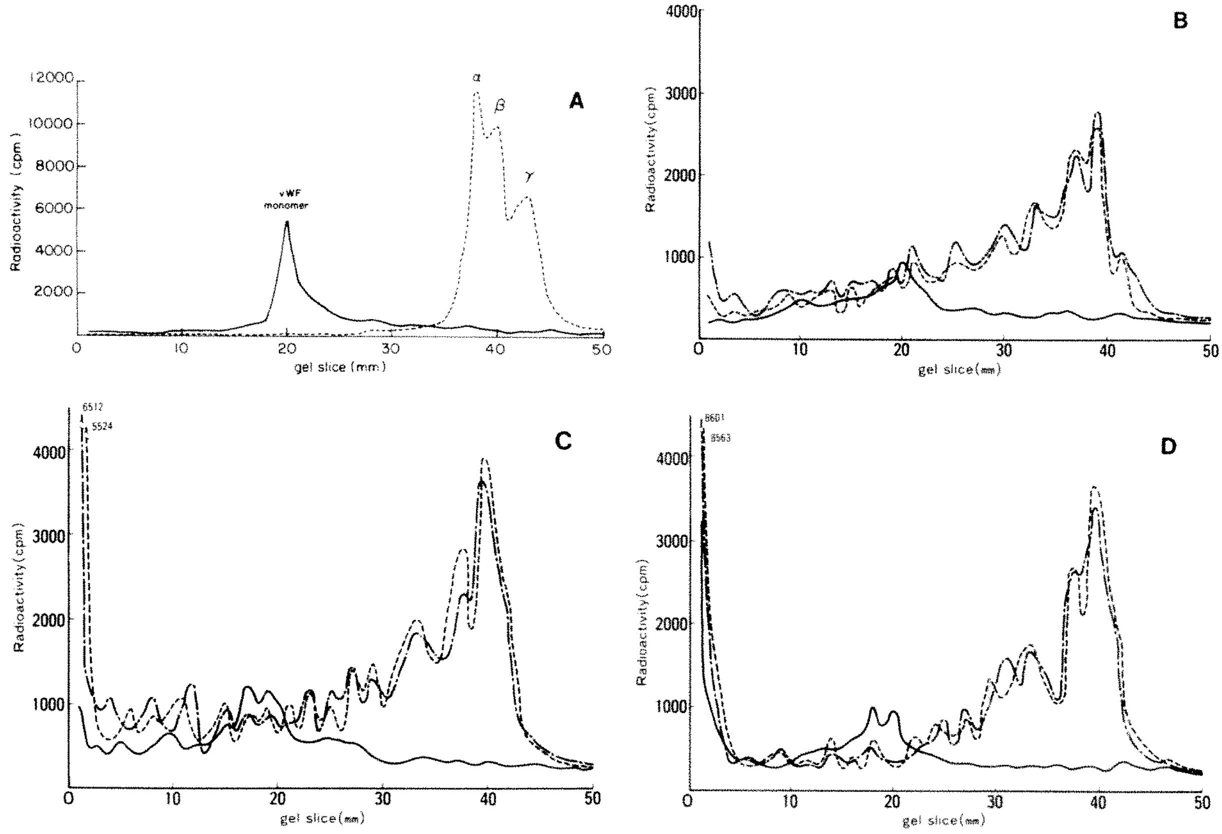

Fig 4. Effect of vWF on fibrin crosslinking. Samples were prepared as follows: (A) ^{131}I-fibrinogen and ^{125}I-vWF were mixed together to show the control proteins; (B through D) samples were prepared in three ways—^{131}I-fibrinogen + vWD, fibrinogen + ^{125}I-vWF, and ^{131}I-fibrinogen. In each case with vWF, the molar ratio was 30 fibrinogen:1 vWF. In all cases, the samples were clotted with thrombin, $CaCl_2$, and 5 U/mL of factor XIII. At intervals, the crosslinking reaction was stopped; samples were analyzed by sodium dodecyl sulfate gel electrophoresis, followed by measurement of radioactivity in the gel segments as in Fig 2B to determine the crosslinking patterns of fibrin without vWF (-·-), fibrin with vWF (----), and vWF (——). (A) Control gel showing starting fibrinogen and vWF: no reaction with factor XIII. (B) Reaction time, 2 minutes. Several fibrin peaks can be identified; from right to left, these represent γ chain (mol wt 48,000), β chain (mol wt 54,000), α chain (mol wt 64,000), γ dimer (~mol wt 96,000), and α dimer (~mol wt 139,000), α trimer (~mol wt 215,000 to 231,000). Larger oligomers cannot be identified with certainty. High-mol-wt α polymer is indicated by the rising counts at the left side (top) of the gel. (C) Reaction time, 15 minutes; (D) reaction time, 240 minutes. In C and D, free γ chain has disappeared, but other lower mol wt peaks are the same as in B.

Table 1. Incorporation of vWF Into Fibrin During Clotting of Normal Plasma and Hemophilia A Plasma

	Normal	Hemophilia A
Recalcification time	10 min	69 min
Plasma fibrinogen	7.3 μmol/L	7.9 μmol/L
Fibrin in gel	92%	76%
Plasma vWF	0.06 μmol/L	0.08 μmol/L
vWF in gel	3%	20%

Covalent crosslinking of vWF to fibrin was also investigated in a plasma test system. Table 1 shows that upon recalcification of normal plasma little vWF became incorporated into the fibrin gel. However, recalcification of plasma from a patient with severe hemophilia A resulted in incorporation of 20% of the plasma vWF. The same results were also obtained with factor IX-deficient plasma, indicating that this was not a special property of hemophilic vWF. In these studies, plasma levels of fibrinogen, vWF, and factor XIII were normal, and the only significant differences were the long recalcification times and increased fibrin-vWF crosslinking in the deficient plasmas. This observation was further investigated by adjusting normal and factor VIII-deficient plasmas to the same clotting times and measuring vWF incorporation in the fibrin gels (Fig 5). Normal plasma was clotted with 10 mmol/L of $CaCl_2$ and dilutions of hirudin and deficient plasma with $CaCl_2$ and dilutions of thrombin so that clotting times of the two plasmas were approximately equal. The results showed that when the clotting times were the same, the amount of vWF incorporation into fibrin was also the same. The distribution of vWF in the plasma clots was examined on reduced SDS gels. In the normal plasma clot (recalcification time 10 minutes), it can be seen that very little vWF was incorporated (Fig 6A). The expected distribution of crosslinked fibrin components was also seen, with predominantly α chain, γ-γ dimer, and high-mol-wt α polymer. Smaller amounts of α monomer and α chain oligomers were also observed. With the factor VIII-deficient clots (recalcification time 69 minutes) vWF was found in the α polymer fraction (Fig 6B). Monomeric vWF and vWF-α chain dimer could also be clearly identified on the gels. The content of α polymer was decreased, and the amount of intermediate α chain oligomers was increased. Thus, in factor VIII-deficient plasma or in normal plasma in which the clotting time was prolonged, vWF was covalently bound to fibrin in a manner identical to that observed with purified reagents.

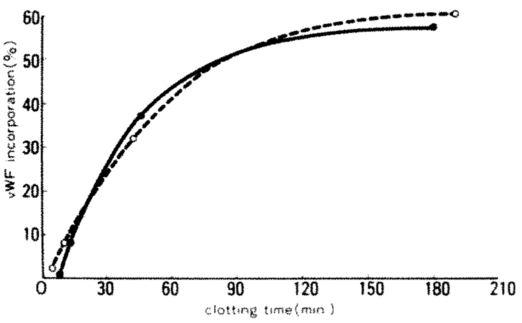

Fig 5. Covalent crosslinking of vWF to fibrin in normal plasma (——) and factor VIII deficient plasma (----) as a function of clotting time. Clotting times were adjusted by adding hirudin to normal plasma and thrombin to deficient plasma. Clots were allowed to form for 3 hours at 37 °C before analysis.

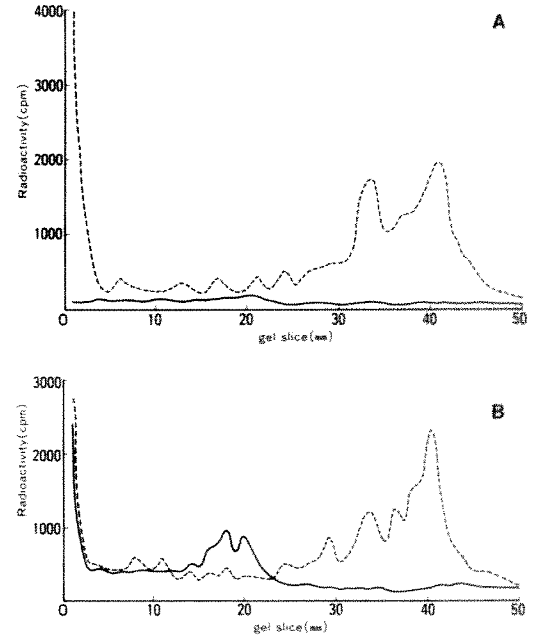

Fig 6. Crosslinking patterns of fibrin and vWF in (A) normal plasma, and (B) factor VIII-deficient plasma. Trace amounts of ^{125}I-vWF and ^{131}I-fibrinogen were added to each plasma and clotting was initiated by addition of 10 mmol/L of $CaCl_2$. Clots were allowed to form for 3 hours at 37 °C before analysis. Fibrin (---); vWF (——).

DISCUSSION

The product of the crosslinking reaction catalyzed by factor XIIIa is ϵ-(γ-glutamyl)lysine, which results from formation of a peptide bond between the γ-carboxamide of peptide-bound glutamine and the ϵ-amino of lysine, thus forming a covalent bridge which links two proteins together.[27] Factor XIIIa appears to have significant substrate specificity requirements, and only certain glutamine and lysine residues in certain proteins are suitable substrates. These include the crosslinking of fibrin γ chains to form γ-γ dimers and fibrin α chains to high-mol-wt α polymers.[28,29] Fibronectin and α_2-antiplasmin are crosslinked to fibrin α chains,[30-32] and fibronectin can also crosslink to collagen.[33] The studies described here show clearly that both monomeric and polymeric forms of von Willebrand factor also act as substrates for factor XIII activity by providing appropriate glutamine residues for the crosslinking reaction. Crosslinking of vWF to fibrin α chains can occur readily during clotting.

vWF is present in normal plasma as a series of disulfide-bonded polymers that range in molecular size from 800,000 to >12,000,000.[34] In these studies, several forms were used: purified vWF monomers, purified polymers with the size distribution of plasma vWF, and the array of polymers distributed in plasma. Purified monomers and polymers, as well as plasma vWF, could participate in crosslinking; but only monomers could clearly crosslink to other monomers to form vWF dimers, trimers, and higher polymers (Fig 2A). Disulfide-bonded polymers of vWF were not crosslinked by factor XIIIa to form larger aggregates (Fig 2B). However, vWF polymers were readily crosslinked to fibrin. In these experiments, it was not ascertained whether the size distribution of polymers which crosslinked to fibrin was random or whether a particular fraction was preferentially involved.

Crosslinking of fibrin γ chains to form γ-γ dimer occurs at the protofibril stage of fibrin gelation.[35] It is rapid, occurs with very low factor XIIIa levels, and is difficult to inhibit.[27] This may be partly because fibrin monomer units are already aligned in the protofibril so that the γ chain crosslinking sites are properly oriented. In contrast, crosslinking of fibrin α chains is slow and highly dependent on the factor XIIIa concentration, with much of the α polymer being formed after gelation.[36] Crosslinking of both fibronectin and vWF to α chains is similar to α polymer formation. In purified test systems with fibronectin, ~50% is crosslinked to fibrin before visible gelation occurs when the thrombin concentration is in the range of 0.05 to 2.0 U/mL, and the remaining fibronectin crosslinking occurs much more slowly.[37] Similar results were observed with vWF-fibrin crosslinking in purified systems, in which prolonged incubation resulted in increased vWF crosslinking after the gel had formed (Fig 4). However, in the plasma system, increased incorporation of vWF polymers was observed when the gelation time was increased (Fig 5), but it was not observed with prolonged incubation after the gel had formed relatively rapidly (Fig 6). The requirement for a long gelation time in plasma may explain why vWF may not be observed as a substrate for factor XIIIa when plasma is clotted rapidly.[38]

Participation of vWF as a substrate for crosslinking by factor XIIIa is similar to fibronectin crosslinking. Each protein can crosslink to itself only in the reduced forms. Both proteins crosslink to the α chain of fibrin and, in so doing, decrease the rate of α polymer formation. However, crosslinking of one protein to α chain does not inhibit the crosslinking of the other. This is similar to the previously described observation that fibronectin and α_2-antiplasmin do not compete for crosslinking sites in α chains of fibrin.[39] It is known that α chains can form at least three covalent crosslinks per polypeptide chain, but this number is not normally obtained in α polymer formation. It appears that some of the lysine crosslinking sites are more readily occupied by glutamines in other proteins than by other α chains.[40] It is unlikely that more than one other protein would be crosslinked to a given α chain because the concentration of α chains in the gel is so much greater than that of other proteins. The principal glutamine crosslinking sites in fibronectin[41] and α_2-antiplasmin[42] are located very close to the amino-termini, well suited for their interdigitation within the α-polymer. A crosslinking site in vWF has not yet been localized.

The observation that vWF polymer formation crosslink to fibrin during plasma clotting (Figs 5 and 6) may explain the decreased level of serum vWF sometimes observed when clotting of hemophilia plasma was prolonged. This was previously ascribed to an alteration in serum vWF.[13] Other investigators have also observed decreased α polymer formation in plasma clots of patients on coumadin or with severe factor VIII deficiency.[43] We interpret the data presented here, as well as the observations of others, to indicate that when the clotting of plasma is prolonged, other proteins, including vWF, can be covalently bound to fibrin α chains by factor XIIIa, with a concomitant decrease in the rate of α polymer formation. It seems likely that this reaction might also be expected to occur in vivo when thrombin generation is limited.

Although the significance of vWF crosslinking to fibrin is unclear, several possibilities can be considered.One possibility involves the contact sites of the fibrin clot with vascular subendothelium. We have obtained evidence that vWF can also crosslink to collagen.[44] Hence, the possibility exists for vWF to function as a covalently held bridge between the fibrin clot and collagen in the subendothelium. The large molecular size of the vWF polymer would be appropriate for such a function. vWF is relatively resistant to plasmin digestion,[45] and its presence in the fibrin clot might also increase resistance of the clot to plasmin degradation. Recent evidence has also been presented concerning the function of vWF in mediating the interaction between platelets and polymerizing fibrin.[46] Covalent crosslinking of a small fraction of vWF to fibrin as it polymerizes would stabilize this interaction. Thrombospondin is also a substrate for factor XIIIa,[47] and it too could have a role in crosslinking reactions involving vWF on the platelet surface.

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Inhibition of Prostaglandin E_2 Restores Defective Lymphocyte Proliferation and Cell-Mediated Lympholysis in Recipients After Allogeneic Marrow Grafting

By Hans-G. Klingemann, Mang-So Tsoi, and Rainer Storb

Prostaglandins are said to influence T and B cell function by inhibiting the generation of interleukin 2 (IL 2) and the formation of suppressor lymphocytes. After bone marrow transplantation, patients usually have a profound immunodeficiency that persists in recipients with chronic graft-*v*-host disease (GVHD) and generally resolves in long-term survivors without GVHD. In vitro tests of lymphocyte function such as allogeneic mixed lymphocyte culture (MLC) and cell-mediated lympholysis (CML) have been shown to be impaired in many patients. We postulated that prostaglandin E_2 (PGE_2) plays a role in the impaired in vitro tests. To test this hypothesis, we studied in vitro tests in the presence of PGE_2 antagonists, indomethacin, and anti-PGE_2 antiserum with cells from 22 short-term patients (<100 days postgrafting) and 32 long-term survivors with or without GVHD. Results show that blockade of PGE_2 release by indomethacin and anti-PGE_2 significantly ($P < .01$) enhanced the MLC (+67%) and the CML responses (+10.5%) of cells from long-term survivors with chronic GVHD but not from those of long-term, stable recipients. No enhancement of MLC and CML activity was observed with cells from donors of long-term recipients. In patients shortly after marrow grafting, enhancement in the MLC was not significant. However, CML activity in this patient group was significantly increased (+12.5% in recipients with no GVHD, 8.5% in those with acute GVHD, $P < .01$). Indomethacin also suppressed the activity of nonspecific suppressor cells in patients with chronic GVHD. When cells from patients with chronic GVHD were treated with recombinant IL 2 and IL 2 combined with indomethacin, it was possible to get an additional augmentation of lymphocyte proliferation after the addition of indomethacin to IL 2–treated cultures. Thus it is very likely that PGE_2 inhibits T lymphocyte proliferation, not exclusively by inhibition of IL2 production or activity. We conclude that PGE_2, among other factors, may play a role in the pathogenesis of the immunodeficiency after transplantation. PGE_2 does not act primarily by interfering with IL 2 but presumably by inducing a suppressorlike activity.

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EXPERIMENTS in a number of species have demonstrated that some immune functions are inhibited by prostaglandin E_2 (PGE_2)[1] such as lymphocyte proliferation,[2,3] activation of cytotoxic lymphocytes,[4,5] cell-mediated lympholysis (CML), and B cell proliferation.[6] In murine and human models, monocytes produce sufficient amounts of PGE_2 to mediate this inhibitory effect.[7,8] There are several explanations for the way in which PGE_2 exerts its inhibitory activity. Evidence is growing that PGE_2 is bound to a functional subset of human T cells that leads to their activation as suppressor cells.[3,9-12] Other data suggest that PGE_2 interferes with interleukin 2 (IL 2) production or activity[13,14] and/or that it reduces Ia expression on monocytes.[15]

Many patients after bone marrow transplantation (BMT) have a profound immunodeficiency related to impaired function of both B and T cells. This immunologic defect may be severe and life threatening, is seen in all patients within the first few months of transplantation, and persists in long-term survivors who have chronic graft-*v*-host disease (GVHD).[16-20] In marrow recipients, reduced lymphocyte proliferation can be restored by exogenous IL 2.[21] The same is true for the diminished CML in patients with acute GVHD, but not for those with chronic GVHD.[22]

It was the aim of this investigation to evaluate whether PGE_2 contributes to the impaired mixed lymphocyte culture (MLC) reactivity and CML of cells from patients after BMT. The results obtained show that in vitro blocking of PGE_2 action by two distinct inhibitors improves lymphocyte proliferation and CML activity in cells from marrow graft recipients. In addition, cells from patients with chronic GVHD were treated with IL 2 or indomethacin, or both. The augmenting effect on allogeneic lymphocyte proliferation by each substance was additive. This observation makes it very likely that PGE_2 inhibits T lymphocyte proliferation not only by inhibition of IL 2 production or activity. Our results point to an indomethacin-sensitive suppressor cell that inhibits lymphocyte proliferation and whose effects cannot be overcome by exogenous IL 2 alone.

MATERIAL AND METHODS

Patients and donors: Marrow transplantation. Cells from 61 allogeneic marrow recipients (8 with aplastic anemia, 38 with acute myelogenous leukemia, 11 with acute lymphoblastic leukemia, and 4 with non-Hodgkin's lymphoma) were studied 22 to 948 days after allogeneic marrow transplantation. The patients' ages ranged from 2 to 49 years; 34 were male and 27 female. Details of the transplantation procedures have been described previously.[23] Patients with aplastic anemia were conditioned for grafting with cyclophosphamide, 50 mg/kg, on each of four successive days. Those with malignancies were conditioned for grafting with a combination of cyclophosphamide, 60 mg/kg, on each of two successive days and total-body irradiation. Evidence for marrow engraftment was obtained by monitoring the peripheral blood count, marrow cellularity, and cytogenetic markers. Patients were divided into four groups based on the time of testing and the presence or absence of GVHD (first figure gives the number of patients tested in MLC; the figure in parentheses refers to the number of patients tested for CML): 11 (10) short-term patients with acute GVHD tested between 22 and 94 days after BMT, 16 (11) short-term patients without GVHD tested

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between 33 and 100 days after BMT, 22 (11) long-term patients with chronic GVHD tested between 294 and 858 days, and 12 (7) long-term patients without chronic GVHD tested between 312 and 948 days after BMT. In all long-term recipients, cells from marrow donors were tested simultaneously with those of their respective recipients to serve as normal syngeneic controls. The protocol for this study was subject to initial approval and subsequent annual reviews by the Human Subjects Review Committee of the Fred Hutchinson Cancer Research Center and the University of Washington. All participants in the study signed committee-approved consent forms.

Reagents. Indomethacin (Sigma Chemical Co, St Louis, catalog No. 1-7378) was dissolved in 95% ethanol at 4 mg/mL and diluted to 20 μg/mL in RPMI 1640 medium (GIBCO, Grand Island, NY) before use. It was determined that addition of ethanol at concentrations equal to those present in the dissolved drug had no detectable effect on our cultures. Indomethacin was used in concentrations of 0.01 μg/mL (3×10^{-8} mol/L) to 10 μg/mL (3×10^{-5} mol/L). Since 1 μg/mL gave optimal enhancement under our experimental conditions as determined by a dose-response curve, we chose this as the final concentration for all experiments added on day 0 to the cultures. Rabbit anti-PGE$_2$ was purchased from Sigma (No. P 6765) and dissolved in phosphate-buffered saline at 5 mg/mL. Lyophilized PGE$_2$ (5Z, 11X, 13E, 15S)-11,15-dihydroxy-9-oxoprosta-5,13-dien-1-oicacid purchased from Sigma (No. P 5640) was initially dissolved in 95% ethanol at a concentration of 0.1 mg/mL (2.8×10^{-4} mol/L) and was used at a final concentration of 10^{-8} mol/L, in RPMI 1640 medium. The final concentration of ethanol in the cultures was <0.1%.

Recombinant IL 2 (lectin free) was purchased from Amgen Biologicals (Thousand Oaks, Calif, No. 01010) and dissolved in 10 μmol/L Tris-buffered saline, pH 8. Twenty units were added to each culture.

Purification of lymphocytes and culture media. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized whole blood by density gradient centrifugation using lymphocyte separation medium (Bionetics) as described.[24] The mononuclear cells were suspended in RPMI 1640 supplemented with 2 mmol/ L-glutamine (GIBCO), 25 mmol/L HEPES buffer (GIBCO), 1% penicillin-streptomycin solution (GIBCO), and 20% heat-inactivated pooled human male serum. All cultures were incubated at 37 °C in humidified 5% CO$_2$ in an air atmosphere.

Mixed lymphocyte cultures. Unidirectional allogeneic MLC were performed in quadruplicate of U-bottomed plates (Costar, Cambridge, Mass) with 1×10^5 responder and an equal number of irradiated (16 Gy) stimulator cells in 0.2 mL RPMI 1640 culture medium. After incubation for six days, cultures were pulsed with 2 μCi of ^{3}H-thymidine (New England Nuclear, Boston) and incubated for six hours. The cultures were harvested and processed with an automated microplate harvester (Cambridge Technology, Cambridge, Mass), and incorporation of radioactivity was measured in a liquid scintillation counter and expressed as the mean counts per minute per quadruplicate cultures ± SD. The variation among microcultures did not exceed 15%. To exclude any direct effect on the responding or stimulator cells, controls were run with responding and stimulator cells using media alone or alternatively with indomethacin, anti-PGE$_2$, or IL 2. Response to allogeneic stimulator cells after addition of indomethacin, anti-PGE$_2$, or IL 2 was expressed as the net counts per minute of cells with the additive minus counts per minute of cells without the additive.

CML assay. To use as few cells as possible from most patients, the indirect CML assay was carried out by a micromethod according to Zarling et al[25] with slight modifications. MLC were set up as described with 2×10^5 responder and stimulator cells per well. Target PBMC (5×10^6/2 mL) from the same donor as the stimulator cells were prepared at the same time as the stimulator and were incubated under the same conditions as the MLC. On day 6 of MLC, the target cells were concentrated in 0.1 mL of heat-inactivated fetal calf serum and were labeled with 300 μCi of ^{51}Cr (Na$_2$ ^{51}CrO$_4$, New England Nuclear) for 60 minutes at 37 °C. After incubation and washing, they were resuspended at 1×10^5/mL in RPMI 1640 with 5% fetal calf serum. An aliquot (0.1 mL) of the supernatant was removed from each MLC culture, and an equal volume of the labeled target cells (1×10^4 cells) was added to each culture, making the ratio of effector to target cells 10:1. After centrifugation (140 g for five minutes) the plates were incubated for six hours at 37 °C in a humidified 5% CO$_2$ incubator.

An aliquot of supernatant was then harvested from each culture and its radioactivity determined in a gamma counter. The percentage of ^{51}Cr release was calculated from the formula: (experimental release − autologous release) × 100/(maximum release − autologous release).

Maximum release values were obtained by adding 0.15 mL of 0.1% Nonidet P40 to 0.1 mL of target cells. Controls with autologous stimulator cells were performed with medium, indomethacin, or anti-PGE$_2$, which were added at the initiation of the assay. In each experiment three different dilutions (5:1, 10:1, 50:1) of effector cells were tested to determine whether a log linear relationship existed between the ratio of effector cells to target cells and the amount of ^{51}Cr release. Since we never observed decreased results at different effector-target ratios (E:T) ratios that did not appear at the 10:1 E:T ratio, only the results obtained with this ratio are presented.

Nonspecific suppressor cell assay. PBMC of the recipient (chimera cells of donor marrow origin) were cocultured with lymphocytes obtained from the marrow donor and irradiated third-party stimulator cells in a mixed lymphocyte reaction. Control cultures consisted of chimera cells alone responding to third-party stimulators and donor cells alone responding to the same stimulators. The percent suppression in any single test was calculated according to the formula: $1 - [DuP/(DuD + PuP)/2] \times 100$ = percent suppression (D, donor PBMC; P, patient PBMC; U, unrelated irradiated lymphocytes). A positive percentage indicated the degree of suppression of donor response by patient cells. A percentage greater than 25% was used as a guideline for detecting cases with suppression.[23]

Statistical methods. The Wilcoxon two-sided signed rank test was applied to paired responses of patient PBMC before and after in vitro treatment. Values for allogeneic induced lymphocyte proliferation from patients were considered significantly different from normal if an individual patient value fell <2 SD below the mean for the control group. Comparisons between groups were done with Student's *t* test.

RESULTS

Improvement of allogeneic MLC of cells from marrow recipients by exogenous indomethacin. Allogeneic lymphocyte proliferation was tested in the MLC with cells from four groups of marrow recipients. Reactivity of cells from patients was compared with that of cells from their respective donors. The results are presented in Table 1 and Fig 1. Cells from short-term patients and patients with chronic GVHD showed a markedly reduced ^{3}H-thymidine uptake as compared with long-term patients without GVHD or healthy donors. Exogenous indomethacin increased the response rate in cells of patients with chronic GVHD ($P < .01$) and in long-term stable recipients ($P < .05$). There was no significant beneficial effect in the cells of patients shortly after transplantation, regardless of whether GVHD was present. There was also no correlation between immunosuppressive medication after marrow grafting (ie, cyclosporine, metho-

Table 1. Effect of Exogenous Indomethacin on Allogeneic MLC of Cells From Marrow Recipients or Donors (Mean cpm ± SE)

Group	n	Indomethacin*	^{3}H-Thymidine Uptake AA†	^{3}H-Thymidine Uptake AB†	Response‡ (%)	Significance§
Short-term patients no GVHD	11	no	197 ± 95	3,697 ± 1,525	100%	
		yes	218 ± 103	3,685 ± 1,255	99%	NS
Acute GVHD	16	no	222 ± 111	6,459 ± 1,931	100%	
		yes	213 ± 100	8,132 ± 2,284	126%	NS
Long-term patients no GVHD	12	no	231 ± 118	11,480 ± 1,933	100%	
		yes	225 ± 113	13,491 ± 2,546	117.5%	$P < .05$
Chronic GVHD	22	no	154 ± 88	6,949 ± 1,310	100%	
		yes	205 ± 79	11,580 ± 1,548	167%	$P < .001$
Marrow donors	28	no	265 ± 109	16,255 ± 1,248	100%	
		yes	244 ± 119	17,214 ± 1,561	106%	NS

*Indomethacin or control medium was added at the initiation of cultures.

†AA, MLC using stimulating cells autologous to responding cells; AB, MLC using stimulating cells allogeneic to responding cells.

‡Percent response = net cpm + indomethacin/net cpm − indomethacin × 100%.

§Significance was assessed by the Wilcoxon two-sided signed rank test; NS, not significant.

trexate, antithymocyte globulin) and impaired MLC responses.

Since indomethacin is not a specific PG synthetase inhibitor, we compared its effect with that obtained with an anti-PGE$_2$ antiserum using cells from eight patients with chronic GVHD. Results in Fig 2 illustrate that ^{3}H-thymidine incorporation in cells from these patients was enhanced by anti-PGE$_2$ in a manner similar to that seen with indomethacin.

Restoration of CML activity with indomethacin in recipients shortly after grafting and in patients with chronic GVHD. CML activity was diminished with cells from all patient groups regardless of the time after transplantation. Results are summarized in Table 2 and Fig 3. Before exogenous indomethacin was added, CML activity was lowest in recipients with acute and chronic GVHD. The addition of indomethacin improved CML by 8.6% and 10.6%, respectively, but normal activity could be achieved only in patients with chronic GVHD and in short-term patients with no GVHD, but not in recipients with acute GVHD. CML activity was also enhanced by indomethacin in long-term patients with no GVHD (+6.3%) and in healthy controls (+4.4%). This improvement was statistically not significant (Wilcoxon signed rank test). As in the MLC, The antiserum against PGE$_2$ was equally effective in enhancing CML activity compared with indomethacin (Fig 2).

Effect of the addition of PGE$_2$. PGE$_2$ (10^{-8} mol/L) suppressed proliferation of cells from healthy individuals in the MLC as well as their activity in the CML (range of suppression, 35% to 68% in both tests, results of eight controls). When indomethacin (1 μg/mL) was added to the cultures together with PGE$_2$, PGE$_2$-induced suppression was not reversed (results not shown).

Additive effects of IL 2 and indomethacin on the allogeneic MLC of patients with chronic GVHD. PBMC of nine patients with chronic GVHD were stimulated with irradiated allogeneic cells in the presence and absence of IL 2, indomethacin, and IL 2 plus indomethacin (Table 3). The addition of IL 2 to PBMC cultures from patients augmented the proliferation by a mean of 60% (range, 7% to 131%). The addition of indomethacin to the same cultures augmented the MLC by a mean of 68% (range, 27% to 156%). However, adding the combination of IL 2 and indomethacin increased proliferation by a mean of 172% (range, 21% to 534%). This increase was statistically significant ($P < .01$) if compared with the increases observed with each single agent.

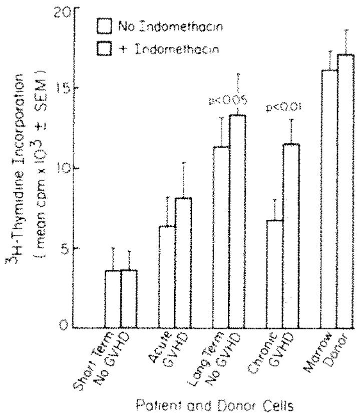

Fig 1. Mixed lymphocyte culture with cells from marrow recipients and donors in the presence and absence of indomethacin. Significance was assessed by the Wilcoxon two-sided signed rank test.

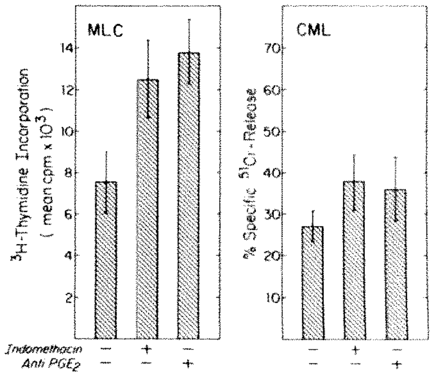

Fig 2. Comparison of the effects of exogenous indomethacin and anti-PGE$_2$ antiserum on the allogeneic MLC and CML of eight patients with chronic GVHD.

Table 2. Effect of Exogenous Indomethacin on CML of Cells From Marrow Recipients or Donors (Percentage of Specific ^{51}Cr Release, Mean ± SE)

Group	n	No Indomethacin	+ Indomethacin*	Percent Increase	Significance†
Short-term patients, no GVHD	9	32.0 ± 7.2	44.5 ± 8.9	12.5	$P < .01$
Acute GVHD	11	16.8 ± 5.1	25.4 ± 5.9	8.6	$P < .01$
Long-term patients, no GVHD	7	29.4 ± 3.5	35.7 ± 5.6	6.3	NS
Chronic GVHD	11	27.0 ± 4.6	37.6 ± 6.7	10.6	$P < .05$
Marrow donors	16	40.9 ± 5.7	45.3 ± 5.9	4.4	NS

*Indomethacin or control medium was added at the initiation of cultures.

†Significance was assessed by the Wilcoxon two-sided signed rank test.

The E:T ratio of CML was 10:1.

Table 3. Augmentation of Allogeneic MLC of Cells From Patients With Chronic GVHD Receiving an Optimal Stimulating Dose of IL 2, Indomethacin, or IL 2 + Indomethacin. The Percent Increase is Given as Compared With the MLC Without Exogenous IL 2 or Indomethacin.

UPN	Underlying Disease	Test Day Postgrafting	Current Medication	Percent Increase of CPM + IL 2	+ Indo	+ IL 2 + Indo
705	AML	2,865	—	40	98	21
1,598	ALL	1,176	P,A	14	61	123
2,113	ALL	628	P	21	27	80
2,337	ALL	416	—	131	94	272
2,361	CML	420	P	117	36	220
2,365	CML	416	P,A	34	29	99
2,386	AML	406	P	79	156	534
2,416	CML	616	P	7	34	83
2,515	CML	261	—	98	79	112
Mean ± SE				60 ± 15	68 ± 14	172 ± 51

Abbreviations: A, azathioprine; P, prednisone; UPN, unique patient number; Indo, indomethacin; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia.

The percent increase of cpm was calculated as follows: (average cpm of indomethacin-treated cells/average cpm of control cultures) − 1.0 × 100.

Generation of nonspecific suppressor cells is decreased after indomethacin. We tested the PBMC of patients with chronic GVHD for nonspecific suppressor cells and found in four recipients nonspecific suppressor activity greater than 25%. Exogenous indomethacin decreased suppression in all of these cases significantly ($P < .001$). In three of these patients suppressor activity was reduced to ≤25% (Fig 4).

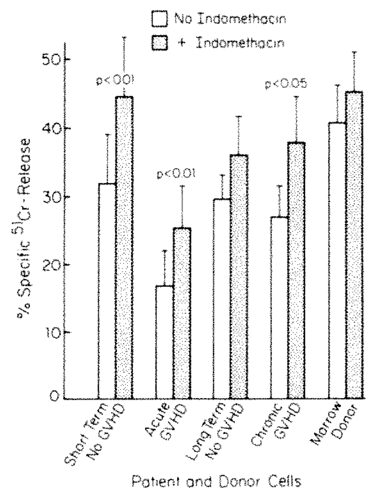

Fig 3. CML with cells from marrow recipients and donors in the presence and absence of indomethacin. Significance was assessed by the Wilcoxon two-sided signed rank test.

DISCUSSION

To understand the underlying causes of immunodeficiency commonly seen in patients early after allogeneic BMT and in long-term survivors with chronic GVHD, we had earlier analyzed various cellular immune functions during different stages of T cell activation of marrow recipients and found that (1) their monocytes usually had normal function[26,27] and could produce IL 1[28]; (2) their T cells were immunologically defective[18,20,28] and were deficient in IL 2 production[21]; and

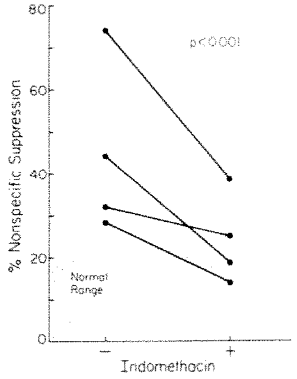

Fig 4. Effect of indomethacin on the activity of nonspecific suppressor cells. Four patients with chronic GVHD demonstrated suppression greater than 25%. In all four cases, suppressor activity was reduced to ≤25%.

(3) exogenous IL 2 restored the T cell CML activity of most recipients early after grafting, but not of patients with chronic GVHD.[22] In addition, nonspecific suppressor cells were found in some recipients, with chronic GVHD[24] thought to contribute to the depressed T cell immunity and subsequently to a higher incidence of late infections.[29] These findings and the reports from others showing that human IL 2 production was regulated by PGE_2[13,14] and that PGE_2 might be involved in suppressor T cell induction[9,11,12,30,31] prompted us to investigate the role of PGE_2 in the immunodeficiency after marrow grafting.

We made use of the findings of several laboratories that PGE_2 was able to inhibit human lymphocyte proliferation[1-4,6] and that blocking of PGE_2 effects by indomethacin or antiserum against PGE_2 could restore the in vitro immune response.[32-34] We were able to demonstrate that the impaired MLC and CML activity with cells from many recipients after marrow transplantation, especially in long-term patients with chronic GVHD, was significantly improved by exogenous indomethacin and also by an anti-PGE_2 antiserum. Our demonstration that a specific anti-PGE_2 serum could mimic the effect of indomethacin suggests that PGE_2, rather than other prostaglandins produced by adherent cells, is the main immunosuppressive factor. These findings suggest a role for PGE_2 in the severe immunodeficiency seen in patients after marrow grafting.

What are the mechanisms of PGE_2-mediated immunosuppression? Some authors believe that PGE_2 directly suppresses human lymphocyte proliferation by interfering with both the production of IL 2 and IL 2–dependent proliferation.[13,14] Others[9,11,32] claim that PGE_2 suppressed lymphocyte responses indirectly by activation of a suppressor cell. Our present data showing that the activity of nonspecific suppressor cells in patients with chronic GVHD can be significantly inhibited by indomethacin points to PGE_2 as a possible inducer of the suppressor activity. Furthermore, Fisher et al[10] recently demonstrated PGE_2 receptors on human suppressor lymphocytes.

As to the cause of increased PGE_2 effects, two explanations are possible: an enhanced sensitivity of grafted lymphocytes to normal PGE_2 levels or an increased production of PGE_2. As shown by Lapp et al,[35] PGE_2 synthesis by mouse macrophages is induced during GVHD reaction. But they also observed that the response to indomethacin was abrogated about 15 to 20 days after GVHD induction. The fact that the augmentation of the allogeneic MLC with indomethacin did not reach significance in our patients with acute GVHD might be explained by this observation, since most of our patients had their disease for more than 2 weeks before being tested.

In addition to our observation with indomethacin only, it was possible to enhance the allogeneic MLC by combining indomethacin and IL 2. The response was superior to the effect of either agent alone. A recent paper by Chouaib et al[36] confirmed this observation. Exogenous IL 2 restored PGE_2-induced inhibition of T cell proliferation only partially. They showed that PGE_2 downregulates the expression of the transferrin receptor, which has been correlated with cell proliferation in normal lymphocytes.

PGE_2 is known to increase the intracellular concentration of adenosine 3'5'-cyclic monophosphate (cAMP).[37] By testing other drugs that enhance intracellular cAMP levels and reduce transferrin receptor expression, Chouaib et al[36] concluded that cAMP might be the mediator of the inhibitory effect of PGE_2 on lymphocyte proliferation. Our observation of a reduction in in vitro activity of nonspecific suppressor cells by indomethacin and the fact that it was possible to get an additional augmentation of lymphocyte proliferation after the addition of indomethacin to IL 2–treated cultures suggest the existence of an indomethacin-sensitive suppressor cell whose effect can apparently not be overcome by IL 2 alone. A similar additive effect of indomethacin and IL 2 was observed in cancer patients whose PBMC were stimulated by phytohemagglutinin.[34,38]

Recent observations in the mouse model showed no increase in the incidence of GVHD by administration of IL 2 in vivo while simultaneously improving the defective immunologic responsiveness of recipient mice.[39] Based on our in vitro studies in man, it might be possible that a combination of IL 2 and indomethacin is superior compared with IL 2 alone in correcting a defective immune response after experimental marrow grafting without aggravating GVHD.

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Cytomegalovirus-Specific Lymphocyte Proliferation and In Vitro Cytomegalovirus IgG Synthesis for Diagnosis of Cytomegalovirus Infections After Bone Marrow Transplantation

By Per Ljungman, Berit Lönnqvist, Gösta Gahrton, Olle Ringdén, and Britta Wahren

With new techniques 19 bone marrow transplant (BMT) recipients were monitored for lymphocyte proliferation and specific IgG production in vitro by cytomegalovirus (CMV) antigen in solid phase. Twelve patients got a reactivated CMV infection as defined by virus isolation or serum IgG conversion. Lymphocyte proliferation and in vitro IgG production responses were significantly stronger in these 12 patients than in seven without ongoing CMV infection ($P = .02$). CMV infection was indicated by the lymphocyte responses at a mean of 45 days after BMT as against a mean of 79 days that passed before CMV growth in culture was detected ($P < .05$). Lymphocyte proliferation and in vitro IgG production may thus be used as tools for diagnosis and for monitoring of CMV infections in BMT recipients.

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CYTOMEGALOVIRUS (CMV) infection is a major problem after allogeneic bone marrow transplantation (BMT).[1] Despite antiviral therapy mortality in CMV, pneumonitis exceeds 80%.[2,3] Treatment with a new antiviral drug, phosphonoformic acid (foscarnet), is reportedly effective in BMT patients with encephalitis and pancytopenia and in renal transplant recipients with pneumonitis.[4,5] In a few cases it has also been effective in BMT recipients with CMV pneumonitis.[6] An early diagnosis of CMV infection is therefore important in these patients. CMV isolations frequently give positive results, but it may take several weeks for a cytopathic effect to appear in culture. Demonstration of early antigens after short-term culture is feasible[7,8] but in our experience may be less sensitive than the classic isolation procedure. Specific IgG and IgM determinations in serum are of minor value for the diagnosis of CMV infections, since in reactivated disease of immunocompromised patients antibody increases often appear late or not at all.[9]

The question whether immunity to herpesviruses is transferred from the donor or persists in the recipient has been addressed. Wahren et al found that the humoral reactivity to viral antigens may either be transferred with donor B cells at BMT or persist among host cells for varying times after BMT.[10] Although engraftment may occur, usually neither long-term transfer nor persistence of herpesvirus-reactive T lymphocytes is seen.[9,11] The cells that mediate the lymphocyte proliferation response to CMV antigen are mainly T cells[12] of the helper/inducer (T4) phenotype (ref 13 and our own unpublished results). Lymphocyte proliferation to all types of antigens, including herpesvirus antigens, is strongly depressed early after BMT.[9,14] Together, these findings indicate that a CMV reactivation should be regarded as a new primary infection by the immune system of the BMT recipient.[14]

New techniques have been developed for measuring specific lymphocyte proliferation to CMV antigen and specific lymphocyte IgG production in vitro.[15] The lymphocytes are exposed to the CMV antigen in solid phase. These techniques were found to be specific and sensitive indicators of reactivity to CMV in healthy individuals. They were found to be useful indicators of CMV infection in BMT recipients.

MATERIALS AND METHODS

Patients. The BMT procedure has been described.[16,17] Out of 20 patients, there were three instances of aplastic anemia, one T cell defect with hypogammaglobulinemia, five acute nonlymphoblastic leukemias (one in first remission, four in partial remission), six acute lymphoblastic leukemias (two in first and four in second remission), one multiple myeloma, and four chronic granulocytic leukemias (two in first chronic phase, one in accelerated phase, and one in second chronic phase (Table 1). All received grafts from HLA-identical, MLC-nonreactive sibling donors. The study was approved by the ethical committee at Huddinge Hospital, and informed consent was obtained from all patients before grafting. The four patients with nonmalignant disease received cyclosporine as prophylaxis against acute graft-*v*-host disease (GVHD). The patients with malignant diseases were randomized between cyclosporine ($n = 10$) and methotrexate ($n = 6$). Cyclosporine therapy was started on day 1 with 5.0 mg/kg/d intravenously (IV) followed by 12.5 mg/kg/d orally in two divided doses when oral medication was tolerated. Acute GVHD was graded according to Thomas et al.[16] Chronic GVHD was defined according to Shulman et al and Schubert et al[18,19] and was verified by biopsies.

Eleven cases in which donor and/or recipient were CMV seropositive before BMT were considered to run a high risk of CMV infection developing.[9] They were included in the randomized study for either prophylactic treatment with CMV-hyperimmune plasma or no such treatment. Lymphocytes from these 16 patients were studied weekly from the time when the white cell count reached 1.0×10^9 cells/L after the initial hypoplasia until 12 weeks after BMT. Thereafter, tests were performed monthly. Four recipients who were seronegative and had seronegative donors were regarded as a low-risk group for CMV infection developing.[9] All these patients received CMV antibody-negative blood products.

Thirteen out of the 16 patients in the high-risk group and all four in the low-risk group survived the first 3 months after BMT. One patient died of a leukemic relapse on day 37 after BMT, and two died of a CMV pneumonitis on day 70 and 75 respectively. After 3 months, one patient died on day 108 of septicemia and one on day 120 of a pneumonitis caused by *Pneumocystis carinii*. Acute GVHD of grade III to IV developed in five patients, grade II in five, and grade I in five. All patients with acute GVHD were treated with prednisone, with an initial dose of 2 mg/kg bodyweight, and seven

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Table 1. Clinical Characteristics, CMV Infections, Lymphocyte Proliferation, and IgG Production Responses in BMT Recipients

UPN	Sex/ Age	Diagnosis/ Remission	CMV Serologic Status		Acute GVHD (Grade)	Chronic GVHD	CMV Isolation (Site)	CMV Symptom	CMV-Specific Lymphocyte Proliferation (net cpm)		CMV-Specific IgG Production (A410)	Death (Day)
			Patient	Donor					Min	Max		
High-risk patients												
L096	M 5	ALL 2nd	pos	neg	III*	no	U + B	fever	1,400	148,000	0.2	131
L100	F 37	CGL 1st	pos	pos	IV*	no	B + L + H U + Br	pneum hepatitis	600	12,000	1.6	75
L099	F 41	ANLL 1st	pos	pos	I†	no	B	none	5,000	55,000		
A016	M 22	AA	neg	pos	0	no	U	none	1,400	12,400	2.0	
L089	M 24	ANLL PR	pos	pos	II†	yes	B + U + BM	panc, fever	0	65,000	2.0	
L098	M 6	ALL 2nd	pos	pos	II†	yes	B	panc, fever	0	49,000	0.8	
A017	M 11	AA	pos	pos	II†	yes	B + U	panc	0	14,000	0.5	
L104	M 34	CGL acc	pos	pos	IV*	yes	B	pneum	500	5,000	0.3	108
L106	M 32	MM	pos	pos	II†	no	B + U + BM	none	0	400	0.9	
L107	M 43	ALL 2nd	pos	pos	III*	no	B + Br	pneum	0	600	0.3	70
L108	M 4	ALL 2nd	pos	pos	I†	no	U	panc	700	12,000	1.4	
L103	F 19	ANLL PR	pos	neg	IV*	no	0	none	300	400	1.0	37
L101	F 37	CGL 2nd	pos	neg	II*	no	0	none	900	3,400	0.4	
L091	M 5	ANLL PR	neg	pos	0	no	0	none	500	1,400	0.1	
H002	F 10	T cell def	pos	neg	I†	no	0	none	0	200	0.1	
Low-risk patients												
A015	M 17	AA	neg	neg	0	no	0	none	300	700	0.1	
L090	M 15	ANLL PR	neg	neg	I†	yes	0	none	0	200	0.1	
L092	M 17	ALL 1st	neg	neg	I*†	yes	0	none	0	800	0.0	
L097	M 41	ALL 1st	neg	neg	II†	no	0	none	100	700	0.05	

Abbreviations: UPN, unique patient number; ANLL, acute nonlymphocytic leukemia; ALL, acute lymphoblastic leukemia; CGL, chronic granulocytic leukemia; acc, accelerated phase; AA, aplastic anemia; MM, multiple myeloma; T cell def, T cell defect; PR, partial remission; B, blood; U, urine; L, lung tissue at autopsy; BM, bone marrow; BR, bronchioalveolar lavage; H, liver at autopsy; 0, CMV not isolated; panc, pancytopenia; pneum, pneumonitis.

*ATG, prednisone, and methylprednisolone.

†Prednisone.

patients received high-dose methylprednisolone and/or antithymocyte globulin in addition (ATG, Table 1).

Diagnosis of CMV disease. The diagnosis of a CMV infection was made by virus isolation on human lung (HL) fibroblasts. Samples for CMV isolation were taken weekly from urine and blood cells during the first 12 weeks after BMT and later at least monthly. CMV isolation attempts were also made on bone marrow and on material obtained from bronchoscopies and liver biopsies and on autopsy material. CMV IgG and IgM antibodies in serum were determined weekly by enzyme-linked immunosorbent assay (ELISA).[20] The following serologic criteria were considered significant for a CMV infection: specific IgM production, CMV IgG seroconversion, or a fivefold specific IgG increase. In patients receiving CMV-hyperimmune plasma transfusions, these were considered in the evaluation of the IgG titer rises.

Viral antigens. CMV nucleocapsid and CMV membrane antigens were prepared from HL cells infected with CMV Ad 169 as described.[20,21] Control antigens were similarly prepared from uninfected HL cells. Lymphocyte proliferation was studied using CMV nucleocapsid antigen because the lymphocytes of healthy CMV-seropositive persons responded better to CMV nucleocapsid than to CMV membrane antigen.[15] CMV membrane antigen was used for stimulation of in vitro CMV IgG production because IgG synthesis in vitro was better with the membrane than with the nucleocapsid antigen. For control purposes, lymphocyte assays were made with herpes simplex virus (HSV) antigens[21,22] and varicella zoster virus (VZV) antigens.[21,23] These tests were sometimes precluded by a lack of cells.

Proliferation technique. Coating of 96-well microplates (Nunc Immunoplate I, Nunc, Roskilde, Denmark) was performed overnight at room temperature with 100-μL volumes (1 μg viral protein) of CMV nucleocapsid antigen. Before use, the plates were washed three times with saline containing 0.05% Tween 20. Patient lymphocytes were prepared from heparinized blood by centrifugation on Lymphoprep (Nyegaard & Co, Oslo). After washing, the lymphocytes were suspended in RPMI 1640 supplemented with 10% CMV antibody–negative AB+ human serum, and 1.5×10^5 cells in 200 μL of suspension were dispersed into two antigen-coated plates. After three and five days, 1 μCi of 3H-thymidine was added. Twenty four hours later the cells were harvested (Titertec, Skatron, Lierbyen, Norway), and the activity was measured in a scintillation counter. Each test was performed in triplicate. A net cpm was calculated as the mean cpm from stimulated cultures minus the mean cpm from control antigen-stimulated cultures. The control antigen was nonstimulatory for the lymphocytes (mean cpm ≤ 800, Fig 1). A net cpm of >1,000 is considered specific for DNA synthesis and indicates that the patient has had a CMV infection.[11,15] A net cpm rise of <10,000 or a more than tenfold rise in the net cpm from a previous test was considered specific for ongoing reactivated CMV infection.

Lymphocyte-produced CMV IgG and IgM determination by ELISA. Microplates were coated with 100-μL volumes (1 μg of viral protein) of CMV membrane antigen.[17] The lymphocytes were suspended in RPMI 1640 with 20% fetal calf serum, and 2.0×10^5 cells were dispersed in duplicate into the antigen-coated plates. After 48 hours the plates were washed three times with saline containing

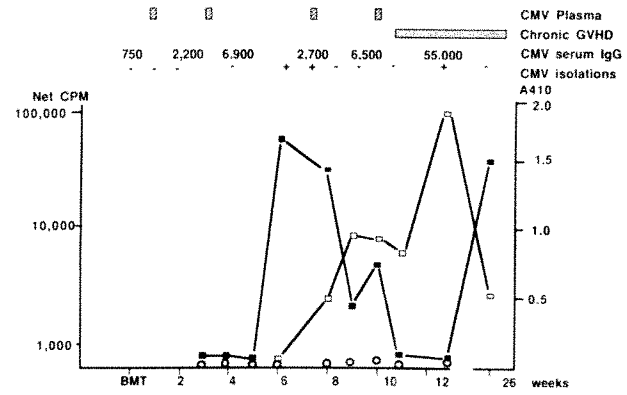

Fig 1. Lymphocyte proliferation to CMV antigen (■), control antigen (O), and specific in vitro IgG production (□) responses after BMT in patient L089 (Table 1) with CMV infection compared with CMV isolation and total specific IgG titers. Loss of responses was followed by a new episode of CMV excretion and a second rise of the proliferation response.

0.05% Tween 20. After washing, 100 μL of alkaline phosphatase-conjugated goat antihuman IgG (Sigma Chemical Co, St Louis) or antihuman IgM (Orion Diagnostika, Helsinki, Finland) was added and the plate incubated for two hours at 37 °C. Then, 100 μL of the substrate *p*-nitrophenyl phosphate (1 mg/mL, Sigma) was added at room temperature, and 30 minutes later the reaction was terminated with 2.5 mol/L NaOH. The absorbance (A) at 410 nm was measured in a Dynatech MR 600 (Arlington, Va). The tests were performed in duplicate. Net absorbance was calculated as the mean A 410 value from CMV-stimulated lymphocytes minus the mean A 410 value of cultures stimulated with control antigen. An A 410 rise of ≥0.4 (0.4 is twice the background level of each assay) was considered to be specific for CMV IgG production.[15,20]

RESULTS

CMV infections. CMV infection developed in 12 of the 16 high-risk patients. Fifteen were confirmed by CMV isolations. Three of them also had serum CMV IgG titer rises. One patient had a CMV IgG seroconversion. None had detectable (titer <50) serum CMV IgM. CMV pneumonitis developed in three patients and two died. The third patient was treated with phosphonoformic acid (foscarnet) and survived the pneumonitis but later died of septicemia. Five patients with CMV infection had pancytopenia and/or fever. Three of the high-risk patients had neither clinical signs of CMV disease nor virologic evidence of a CMV infection. One patient was not evaluable because virus isolations were not adequately sampled. No CMV infection developed in any of the low-risk patients.

Lymphocyte proliferation responses. CMV-specific lymphocyte proliferation responses developed in ten of 12 patients with CMV infections (minimum values for previous tests and maximum cpm values for each patient are shown in Table 1). Eight of these patients had a net cpm of more than 10,000 and all had a more than tenfold rise in the net cpm compared with a previous test (Table 1, Fig 1). None of the seven patients without CMV infection had a net cpm of more than 3,400 (Table 1, Fig 2). Table 2 shows a significant difference in the mean responses between the two patient groups (*P* = .02 by the two-tailed Mann-Whitney U test).

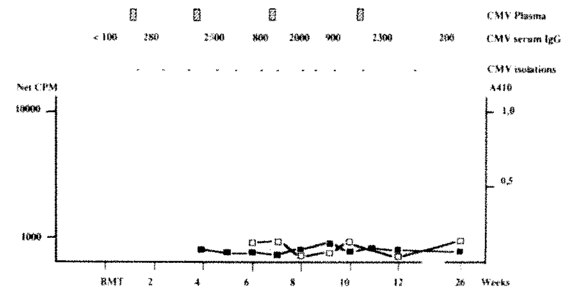

Fig 2. Lymphocyte proliferation (■) and IgG production (□) responses in a patient (L091) without CMV infection (seropositive donor, seronegative recipient).

The proliferation responses rapidly rose to a peak value, and during follow-up the responses usually diminished (Fig 1). In three patients, lymphocyte proliferation responses before treatment with ATG (net cpm of 148,000, 48,000, and 8,000, respectively) became deeply depressed after this treatment (net cpm <1,000). In three out of four patients in whom chronic GVHD developed, a strong CMV-specific proliferation response at the diagnosis of CMV infection was lost during follow-up.

The strong proliferation responses occurred specifically in response to infection. In every case where we found a net cpm of >10,000 after BMT against any herpesvirus antigen (CMV, HSV, VZV), a virus reactivation was confirmed by other means.

Two patients had a herpesvirus antigen-stimulated net cpm of between 5,000 and 10,000 the first weeks after marrow take. This was followed by a rapid decrease. One of these patients (L100) is presented in Fig 3. CMV was later isolated, and a second rise to a net cpm of 12,000 was seen before the patient died of CMV pneumonitis. This patient was CMV-seropositive before BMT and had a seropositive donor. Either the transfer of CMV-reactive lymphocytes from the donor or the persistence of the patient's own lymphocytes would explain the stimulation response directly after BMT. The second patient (L97) was seronegative for HSV before BMT and had an HSV-seropositive donor. This patient had a positive lymphocyte response with HSV antigen without signs of HSV infection during the first 2 months

Table 2. Mean Maximum CMV-Specific Lymphocyte Proliferation and Mean Specific Antibody Production In Vitro for BMT Recipients With and Without CMV Infection

Patients	Mean Net cpm (Range)*	Mean Net A 410 (Range)*
With CMV infection (n = 12)	31,400 (400-148,000)	0.9 (0.2-2.0)
Without CMV infection (n = 7)	900 (200-1,400)	0.2 (0.0-1.0)†

**P* = .02, Mann-Whitney U test, two tailed.

†One patient died early in relapse.

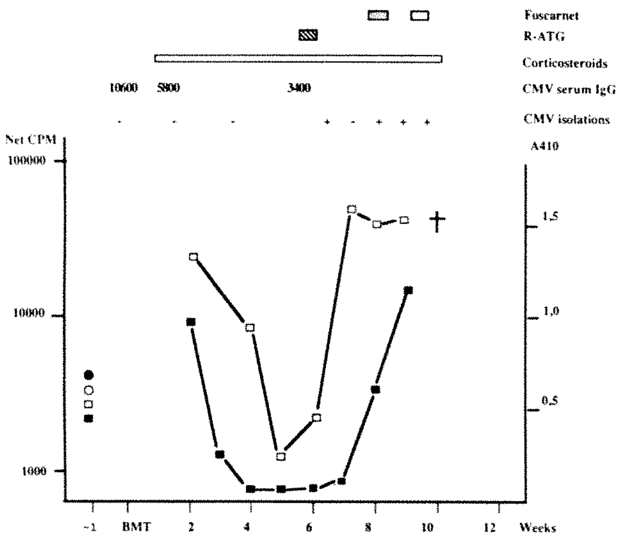

Fig 3. Lymphocyte proliferation (■, patient; ●, donor) and IgG production (□, patient; ○, donor) responses in patient (L100) who died of CMV pneumonitis. In this patient transfer or persistence of CMV-reactive lymphocytes may have occurred (first peak of specific proliferation and IgG responses).

after BMT. The response was lost later. This indicates a transfer of reactive cells from donor to recipient.

CMV-specific antibody production in vitro. Specific IgG production by lymphocytes could be detected (net A 410 > 0.4) in nine of 12 patients with CMV infection. Eight had at least a fivefold rise in the amount of CMV IgG produced as judged by the absorbance levels. In eight of these patients, at least two successive positive tests were found. In six of the seven patients without CMV infection, no IgG production could be detected (A ≤ 0.4; $P < .05$ by the two-tailed Mann-Whitney U test). One patient, who died early in relapse, had an A 410 of 1.0 that later decreased rapidly. No CMV IgG rises were seen in patients without a CMV reactivation. No lymphocyte-produced CMV-specific IgM was detected in any of the patients.

In six patients an initial IgG response to CMV antigen was seen, followed by a decrease (Fig 3). Five of these recipients were seropositive before BMT with seropositive donors. CMV infection developed in these patients, and in three of them, later, strong CMV IgG production was seen. Both transfer and persistence of IgG-producing lymphocytes are feasible in these cases.

CMV-specific lymphocyte proliferation and CMV IgG production in vitro correlated with the diagnosis of CMV infection. Lymphocyte proliferation responses and in vitro IgG production both quickly indicated a CMV infection after BMT (Table 3). When the two methods were combined, 11 of 12 CMV infections were detected. The 12th patient had severe acute GVHD and was treated with methylprednisolone and ATG. He later died of a CMV pneumonitis. A positive CMV-specific lymphocyte proliferation and/or an in vitro IgG production response developed significantly sooner than growth of CMV in culture or a CMV IgG titer rise in serum ($P < .05$ by Wilcoxon's rank sum test). In six of seven patients, either positive CMV-specific lymphocyte proliferation or IgG production was found before either CMV isolation or a serum antibody increase. In the seventh patient, a CMV IgG increase in serum was the first sign of CMV infection.

Table 3. Day for Diagnosis Post-BMT of CMV Infection by Virus Isolation and Serum CMV IgG Compared With the Day for Rise of CMV-Specific Proliferation Response (Net cpm >10,000 or a Tenfold Net cpm Rise) and/or In Vitro Production of CMV IgG (A 410 > 0.4)

Test for Diagnosis of CMV Infection	No. of Patients		Mean No. of Days After BMT (Range)
	Positive	Negative	
Samples taken			
CMV isolation	11	1	45 (33-76)
Serology (>5-fold IgG rise)	4	8	87 (31-122)
Lymphocyte proliferation	10	2	39 (17-72)
In vitro IgG production	9	3	53 (20-80)
Diagnosis obtained			
CMV isolation	11	1	79 (61-112)
Serology + CMV isolation*	12	0	80 (31-123)
Lymphocyte proliferation + in vitro IgG production*	11	1	45 (19-78)

*$P < .05$, Wilcoxon's rank sum test.

DISCUSSION

The magnitude of CMV-specific lymphocyte proliferation responses was found to differ significantly between patients with and without CMV infection. There was also a difference in the in vitro CMV IgG production response. Responses seen early after BMT may reflect transfer or persistence of reactive lymphocytes, but such early responses usually diminished during the first weeks. Patients with proven reactivated CMV infections had secondary strong rises of in vitro lymphocyte responses to CMV. A strong rise in the lymphocyte proliferation response and/or a rise in the production of CMV-specific IgG in vitro may therefore be used as adequate diagnostic signs of a CMV infection.

We used unfractionated lymphocyte populations to minimize the loss of cells with specific functions, especially those of antigen presentation. This also has the advantage that the T cell and B cell responses can be analyzed simultaneously.

It is difficult to define exactly the time after BMT at which a CMV reactivation starts. We therefore calculated the number of days from BMT until the first positive assay for CMV was obtained with each of the methods. With the criteria used (a net cpm rise to >10,000, a tenfold rise in net cpm, or a net A 410 rise of >.4), the time to diagnosis of a CMV infection was determined by the time at which first positive CMV isolation was obtained. Considering the time required for CMV replication in culture and the in vitro lymphocyte tests, the detection of CMV reactivation was achieved significantly sooner with the lymphocyte proliferation/IgG production tests.

We noted that CMV-specific lymphocyte proliferation responses reached an early peak and often diminished later. The reason for this is unknown. It may have to do with a decreased activity of T4 cells. CMV infection has been associated with a depressed T4/T8 ratio,[24] and in four of seven patients with CMV infection, a low T4/T8 ratio was found (data not shown). Patients who received ATG treatment rapidly acquired a depressed lymphocyte proliferation response to CMV. A decreased activity of T4 cells may be mediated by suppression, for instance, induced by CMV. In two of three patients in this study in whom chronic GVHD has thus far developed, the lymphocyte proliferation responses were lost while CMV excretion has continued. This is in agreement with our earlier results showing that lost CMV-specific lymphocyte proliferation responses were more common in patients with chronic GVHD.[9] Chronic GVHD is associated with immunoincompetence,[25,26] and nonspecific suppressor cells have been found in patients with chronic GVHD (for a review see Tsoi et al, ref 27).

In summary, we have found that increased CMV-specific lymphocyte proliferation and in vitro IgG production to CMV antigen may be used as tools for the early diagnosis of CMV infection after BMT.

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Expression of p53 in Human Leukemia and Lymphoma

By M. Prokocimer, M. Shaklai, H. Ben Bassat, D. Wolf, N. Goldfinger, and V. Rotter

Analysis of fresh human tumors have indicated that patients with B type lymphoproliferative diseases and the majority of patients with acute lymphoblastic leukemia (ALL) express elevated levels of p53 production. It is suggested that in these human malignancies, p53 may provide a novel tool for monitoring cancer activity. Conversely, p53 is not expressed in acute myeloid leukemias, myeloproliferative diseases, or myeloid leukemic cell lines. Analysis of the p53 gene structure indicated the existence of similar patterns of p53 restriction fragments in producer and nonproducer cells, which suggests that the p53 gene is not altered in the latter. However, in one case of acute promyelocytic leukemia (APL), we have observed a rearrangement in the p53 gene. Karyotype analysis has indicated that these APL cells do not contain the typical 15;17 translocation. In other APL patients who exhibit a 15;17 translocation, we found no genomic changes of the p53, suggesting that the p53 gene, which was recently mapped to the short arm of chromosome 17 in the human, is not structurally related to the typical chromosomal break point found in the long arm of chromosome 17 of APL patients.

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THE CELLULAR encoded protein p53 is overproduced in cancer cells. Accentuated concentrations of this protein have been detected in a large number of transformed cell lines[1] and in primary tumors in mice.[2] It was found in a variety of tissue types, including sarcoma and leukemia cells of several species.[3-7]

A direct role for p53 in the process of carcinogenesis was suggested by experiments that showed that expression of the p53 gene can immortalize cells and that the p53 gene can replace the *myc* oncogene in a *myc-ras* immortalization/transformation assay.[8-10] In addition, we found that the introduction of a functional p53 gene into p53 nonproducer[11] L12 Ab-MuLv–transformed cells that have a rearranged p53 gene changed their phenotype from cells that induce regressor tumors into cells that induce the appearance of lethal tumors. These and other experiments suggest a correlation between expression of p53 in tumor cells and the capacity of the latter to exhibit a fully transformed phenotype.

Recently we and others became interested in evaluating the possible role of the p53 oncogene in human neoplastic growth and differentiation. We were especially interested in studying the expression and gene structure of p53 in human leukemia-lymphoma cells in comparison with analogous normal tissues. A study of the hematologic malignant diseases was attractive because we have recently mapped the p53 gene to the short arm of chromosome 17.[12] Gain or loss of the whole chromosome 17 or parts of it has been a frequent finding in these malignancies.[13] We were in particular interested in studying patients with acute promyelocytic leukemia (APL), since a reciprocal translocation involving chromosomes 15 and 17 at bands q22 and q21, respectively, is a characteristic abnormality in APL.[14] The study group consisted of patients with (1) acute leukemias, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL); (2) myeloproliferative diseases, chronic myeloid leukemia (CML) with Philadelphia chromosome–positive cells (ph'+), myelofibrosis (MF), myeloid metaplasia (MM), and polycythemia vera (PV); (3) chronic lymphocytic leukemia (CLL); and (4) lymphoma in leukemic phase. We extended our study to include a large number of established human lymphoma-leukemia cell lines. The availability of a cloned human p53 cDNA[15] and specific anti-p53 monoclonal antibodies[16-18] permitted us to survey p53 expression and its gene structure in human lymphoma-leukemia cells.

MATERIALS AND METHODS

Cell lines. The following human leukemia-lymphoma cell lines were tested: ALL cell lines including Reh, Km-3, Peer, Amsalem, Molt-4, Nalm-6, Nalm-1; Burkitt's lymphoma cell lines including Ramos, Bjab, Bjab-B958, Ra-B958, Raji, Daudi; Myeloid cell lines[19] including the K-562 erythroleukemic cell line,[20] HL-60 promyelocytic cell line,[21] and the Dekabuto myeloid cell line (Ben Bassat, unpublished observations). In addition we tested SV80, a human transformed fibroblastic cell line expressing the SV40 DNA tumor virus,[22] and a normal human permanent B cell line established from peripheral blood lymphocytes infected by Epstein-Barr virus (EBV) (B958) (Ben-Bassat, unpublished observations).

All cell lines were grown in RPMI 1640 enriched with 10% heat-inactivated fetal calf serum (Biolab, Jerusalem, Israel) and 2×10^{-5} mol/L β-mercaptoethanol. Hybridoma cell lines PAb421,[16] PAb122,[17] and RA3-2C2[18] were grown in RPMI 1640 medium enriched with 20% heat-inactivated fetal calf serum supplemented with 20 mmol/L L-glutamine and 20 mmol/L sodium pyruvate.

Patients. Samples of heparinized venous blood and bone marrow cells were obtained from patients and normal healthy volunteers who gave informed consent. The samples were collected at the time of diagnosis or at relapse from patients with ALL, AML, CML ph+', MF, lymphoma in leukemic phase, and CLL. Diagnoses were made by using standard clinical, morphologic, cytochemical, and immunologic criteria. Eighty percent or more of the cells in each of these cases was malignant by the aforementioned criteria.

Antibodies. Monoclonal anti-p53 antibodies were obtained from the established hybridoma cell lines PAb421,[16] PAb122,[17] and RA3-2C2.[18] Monoclonal antibodies were obtained from either supernatants of the hybridoma cell lines or ascitic fluid of syngeneic mice

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injected intraperitoneally (IP) with the hybridoma cell lines. Antibodies were purified and concentrated by binding to Sepharose–protein A columns (Sigma Chemical Co, St Louis).

Synthesis of p53 protein. A quantity of 1×10^7 cells of each individual cell line, sample of bone marrow, peripheral blood mononuclear cells, or one plate of fibroblasts at the logarithmic stage of growth were washed several times in phosphate-buffered saline (PBS), resuspended in 1.5 mL Dulbecco's modified Eagle's medium without methionine, and enriched with 10% dialyzed heat-inactivated fetal calf serum and 250 μCi ^{35}S-methionine (purchased from Amersham Corp, Buckinghamshire, England). Cells were incubated for one hour at 37 °C, washed in PBS, and extracted into 2 mL of lysis buffer (10 mmol/L Na_2HPO_4-NaH_2PO_4, pH 7.5, 100 mmol/L NaCl, 1% Titron X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate) at 4 °C. Labeled cell lysates were precleared by repeated absorption on *Staphylococcus aureus* and nonimmune serum. Equal amounts of radioactive protein were immunoprecipitated with specific antibodies. Antigen-antibody complexes were collected by *S aureus*.[23] Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli.[24]

Southern blot analysis. Genomic DNA was prepared from individual cell lines and fresh leukemia-lymphoma cells. Cells were washed twice with PBS, resuspended in lysis buffer (0.5% SDS, 50 mmol/L Tris-HCl, 5 mmol/L EDTA, pH 7.5) and 0.2 mg/mL proteinase K (Boehringer Mannheim, FRG), and incubated for 14 hours at 37 °C. The solution was extracted twice with an equal volume of phenol and twice with equal volumes of chloroform/isoamylalcohol 24/1. The DNA solution was adjusted to a final concentration of 0.3 mmol/L Na-acetate, and 2 vol of cold ethanol was added. The DNA precipitate formed at room temperature was washed twice with 80% ethanol and resuspended in 10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 7.5). Aliquots of 10 μg DNA digested with *Eco*RI and *Hind*III were electrophoresed on 0.8% agarose gel, blotted onto nitrocellulose filter,[25] and hybridized to nick-translated[26] p53 DNA probes.[15,27]

RESULTS

Expression of p53 protein in established human cell lines. Using the human specific anti-p53 monoclonal antibodies PAb421 and PAb122, we screened a large number of human lymphoma-leukemia cell lines for expression of p53. Figure 1 illustrates an example of several human cell lysates immunoprecipitated with PAb421. As shown in this figure, human cell lines express more than one p53 protein species. Some lines, like SV-80–transformed fibroblasts and the Reh-ALL cell lines, synthesize two physically distinct proteins, whereas others express either the lower or the upper p53 protein species (Molt-4 and Nalm-6, respectively). Table 1, which summarizes results with all the human lines we have studied, indicates that all T and B leukemia cell lines as well as a normal human permanent B cell line infected by EBV B958 expressed the p53 protein evaluated by immunoprecipitation with specific anti-p53 monoclonal antibodies. These findings are in agreement with the detection of the p53 protein in human cell lines previously documented.[5,6] p53 synthesis, however, was not detected in the following human myeloid leukemic cell lines: HL-60,[21] K-562 (Fig 1), and Dekabuto (not shown).

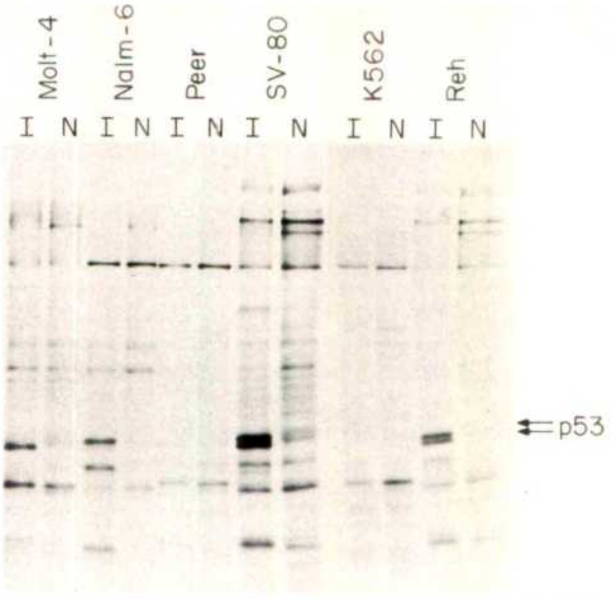

Fig 1. Expression of the p53 protein in human established cell lines. Equal amounts of ^{35}S-methionine–labeled cell lysates were immunoprecipitated with nonimmune serum (N) or PAb421 anti-p53 monoclonal antibodies (I). The dried gel was autoradiographed for 1 week.

Analysis of the p53 gene in the p53 producer and unproducer cell lines tested by Southern blotting using a human specific cDNA probe, p53-H-13,[15] indicated a similar pattern of restriction fragments digested with EcoRI or double digested with *Eco*RI and *Hind*III in all cell lines. The HL-60 cells are the only exception; they were found to contain a major deletion in the p53 gene (Fig 2).[27]

Expression of p53 in fresh leukemia-lymphoma cells. Fresh human leukemia-lymphoma cells were tested for expression of the p53 protein using human-specific anti-p53 monoclonal antibodies, PAb421,[16] and a control of

Table 1. Expression of p53 in Established Human Lymphoma-Leukemia Cell Lines

Cell Line	Origin	p53 Expression	Properties of p53 Antigens
Reh	N-ALL	+	D
Km-3	N-ALL	+	D
Peer	T-ALL	+	S
Molt-4	T-ALL	+	S
Amsalem	T-ALL	+	S
Nalm-6	pre-B-ALL	+	D
Daudi	BL	+	S
Raji	BL	+	D
Ramos	BL	+	S
Ra-B958	BL	+	S
BJAB	BL	+	S
BJAB-B958	BL	+	S
K-562	CML-BC	—	—
Nalm-1	CML-BC	+	S
HL-60	APL	—	—
Dekabuto	AML	—	—

Abbreviations: BL, Burkitt's lymphoma; CML-BC, CML in blast crisis; S, single p53 protein; D, doublet of p53 protein; +, p53 detected in ^{35}S-methionine–labeled cell extracts by immunoprecipitation with human specific anti-p53 monoclonal antibodies PAb421 and PAb122; —, p53 undetected by immunoprecipitation.

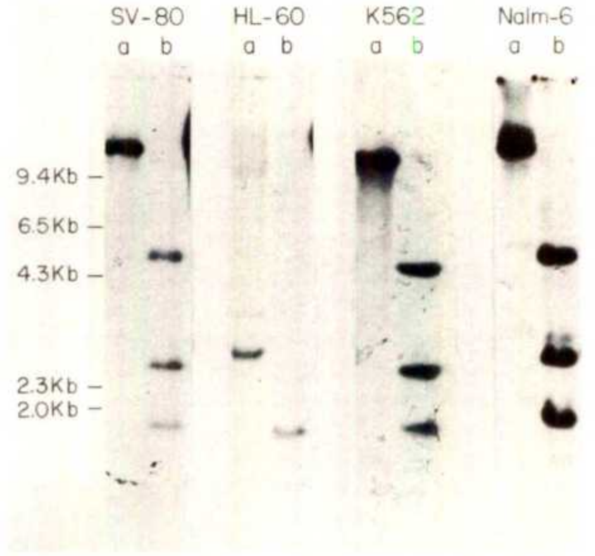

Fig 2. **Southern blot analysis of p53 producer and nonproducer human cell lines. Equal amounts of DNA digested with *EcoRI* (a) or double digested with *EcoRI* and *HindIII* (b) were separated by electrophoresis and hybridized with a human-specific cDNA probe, p53-H-13.[15]**

Table 2. Expression of p53 in Fresh Human Lymphoma-Leukemia Cells

Diagnosis	No. Studied	No. Positive
B-CLL	19	19
B lymphoma in leukemic phase	7	7
ALL	5	4
AML*	17	—
CML-ph' +	11	—
MF	10	—
PV	4	—

*The study group includes M_{1-6} morphologic subtypes (French-American-British classification, FAB[28].

(—), p53 was undetectable by immunoprecipitation.

RA3-2C2, mouse-specific anti-p53 (Fig 3).[18] p53 expression was detected in all of the following patients: (1) B-CLL, (2) B lymphoma in leukemic phase, and (3) in five out of six patients with ALL (Table 2). Overall, ALL and lymphoma samples displayed more intense signals than did the CLL samples. In the latter, prolonged exposures of 3 to 4 weeks' duration were usually required for visualization of the signals. As in human cell lines, fresh tumors express more than one p53 species. Indeed, we observed several tumors that synthesize a single p53 protein, whereas in others, two and occasionally three physically distinct proteins were detected.

Evaluation of p53 expression in nontransformed human cells indicated the absence of detectable levels of p53 in thymocytes, in peripheral blood lymphocytes, and in bone marrow progenitor cells. p53 protein synthesis, however, was detected in fetal cord blood obtained immediately after delivery.

p53 synthesis was not detected in patients with AML, CML (ph'+), MF, or PV (Table 2). Prolonged exposures of autoradiograms for longer than four weeks, did not yield a p53 signal in these samples.

Southern blot analysis of genomic DNA isolated from these samples was performed using the p53 human-specific cDNA probe (p53-H-13).[15] The results illustrated in Fig 4 showed a similar pattern of restriction fragments in producer and nonproducer cells, suggesting that the p53 gene analyzed

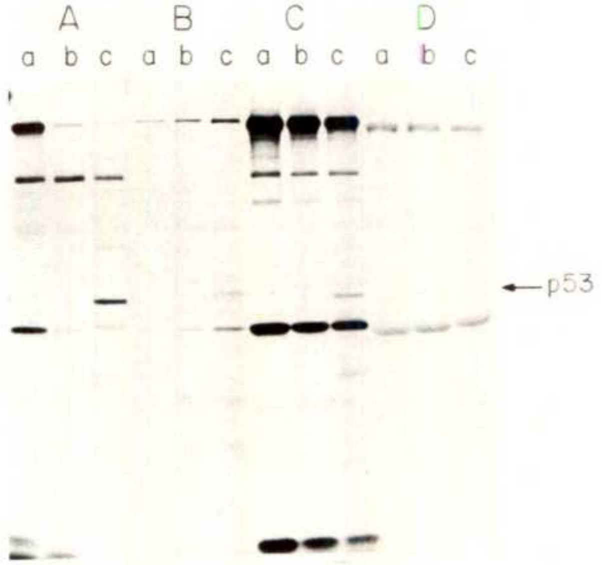

Fig 3. **Expression of p53 protein in human fresh leukemia-lymphoma cells. Patients with the following diseases were studied: lymphoma (A), CLL (B) ALL (C), and AML (D). Equal amounts of ^{35}S-methionine–labeled cell lysates were immunoprecipitated with either nonimmune serum (a), RA3-2C2 mouse-specific anti-p53 (b), or PAb421 human-specific anti-p53 (c). The dried gel was autoradiographed for 4 weeks.**

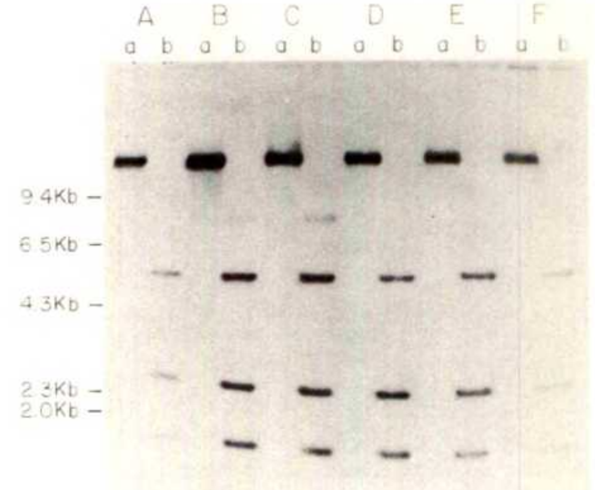

Fig 4. **Southern blot analysis of the p53 gene in fresh leukemia and lymphoma samples. DNA analyzed was obtained from patients with the following diseases: A, B, lymphoma; C, D, CML; E and F, AML. Equal amounts of DNA (10 μg), digested with *EcoRI* (a) or double digested with *EcoRI* and *HindIII* (b) were separated by electrophoresis and hybridized with the human-specific cDNA probe p53-H-13.[15]**

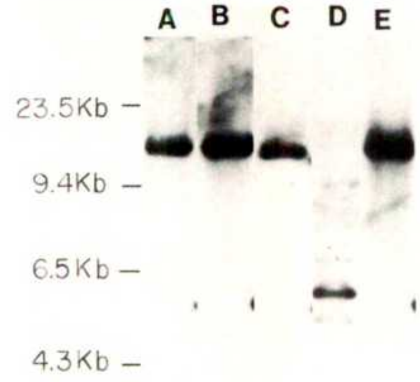

Fig 5. Southern blot analysis of the p53 gene in patients with APL. Equal amounts of DNA obtained from individual patients were digested with *EcoRI*, separated by electrophoresis, and hybridized with the human-specific cDNA probe p53-H13. Karyotype analysis of patients A, B, C, and E exhibited the classic 15;17 translocation. Karyotype analyses were performed on G-banded preparations as previously described.[32,33]

under these resolution conditions is intact in p53 nonproducer fresh tumor cells.

Analysis of the p53 gene in leukemic cells of patients with APL. Previously we found that most of the p53 gene in the HL-60 promyelocytic cell line was deleted.[27] In these cells only one chromosome 17 was detected.[29] The fact that the human p53 gene was shown to map to chromosome 17 as well as the observation of Larson et al showing a consistent 15q22 to 17q21.1 chromosomal translocation in patients with APL[14] prompted us to extend our survey and analyze p53 expression in APL patients. To that end five patients with APL were analyzed. The criteria for the diagnosis of APL were as described previously.[31] All patients either had disseminated intravascular coagulation on presentation, or the syndrome developed after initiation of chemotherapy. Cytogenetic evaluation of bone marrow was performed in all the patients. Four patients had the classic translocation 15;17, and one had a normal karyotype. Results shown in Fig 5 indicated that four patients who had the classic translocation seem to present an intact p53 gene as evaluated by Southern blotting. A typical pattern of restriction fragments was obtained with these patients (Fig 5, lanes a, b, c, and e). However, in the single patient diagnosed as having APL exhibiting a normal chromosomal karyotype with no 15;17 translocation, a rearrangement of the p53 was observed (Fig 5, lane d).

DISCUSSION

Recently we and others became interested in studying p53 expression in human cancers. These studies have been directed toward the question of whether the p53 gene is overexpressed in human malignancies.

Our study has focused on evaluation of the p53 protein expression and its gene structure in the fresh human lymphoma-leukemia cells and in established lymphoma-leukemia cell lines. Using the human-specific anti-p53 monoclonal antibodies PAb421, PAb122, and a cloned human p53 cDNA as a probe, we screened a variety of human primary tumors. We have observed that most of the malignant tissues studied expressed an elevated level of p53 synthesis compared with analogous normal tissues. All Burkitt's lymphoma and ALL (T and B) human cell lines as well as all patients with B-CLL, B lymphoma in leukemic phase, and a majority of patients with ALL expressed elevated levels of p53 protein synthesis. This suggests that p53 overexpression is an authentic phenomenon rather than a tissue culture artifact.

We found that fresh ALL, B lymphomas generally displayed an increase in signal intensity compared with the majority of CLL cells, suggesting that the rate of synthesis of p53 in leukemic cells derived from patients with ALL lymphomas is increased over that in CLL cells. Since p53 is a nuclear-associated protein that may be involved in the control of DNA replication, it is possible that p53 is more frequently synthesized in the more rapidly proliferating acute leukemia or lymphoma cells than in the less-proliferating CLL cells. Analysis of the p53 protein in fresh tumors has indicated the presence of several p53 protein species varying in their molecular size. As in human cell lines, some synthesize a single p53 protein, whereas others synthesize two or even three physically distinct proteins. This differential expression of several p53 species by the single human p53 gene could be controlled by an alternative splicing mechanism (manuscript in preparation).

Previously we observed that the p53 gene of HL-60 cells, a promyelocytic leukemic cell line, was significantly altered. Most of the p53 gene was deleted in these cells and therefore failed to produce the p53 protein.[27] In this study we further observed the lack of a detectable level of p53 protein in other myeloid cell lines such as the K-562 (erythroleukemic cell line derived from the pleural effusion of a patient with CML in blastic crisis) and Dekabuto cell lines. These findings are in agreement with the results reported by Miller et al.[34] It should be noted, however, that p53 is expressed in the Nalm-1 cell line.[35] This line was established from the peripheral blood of a patient who was in blastic crisis of ph^1 CML. It was derived from the lymphoid type of CML blasts. Lack of p53 expression was also evident in fresh samples obtained from patients with AML and myeloproliferative diseases such as CML, MF, and PV. These observations suggest that this gene may play a role in early hematopoietic differentiation. The p53 gene may be completely shut off through gene inactivation in myeloid differentiation. Alternatively, lack of the p53 protein in myeloid leukemias might suggest the existence of a different transformation mechanism in these malignancies.

Southern blot analysis of p53 producer and most of the nonproducer cell lines or fresh tumors studied here indicate that the p53 gene seems intact. Under the present resolution conditions, we have observed similar patterns of p53 restriction fragments in p53 producer and nonproducer cells.

In the group of APL patients, we observed one of the five in which the p53 gene was rearranged. In that particular case no 15;17 translocation typical for APL was evident. In the other four cases of APL in which a typical translocation of the long arm of chromosome 17 to chromosome 15 was found, no modifications in the p53 gene were observed. This strongly suggests that the 15;17 translocation break point and the p53 gene are not structurally related. This is further emphasized by the fact that the p53 gene was mapped to the short arm of chromosome 17, which is usually not altered by the typical 15;17 q22-21.1 translocation.[12] Taken together, it seems that the assumption that p53 has a critical involvement in the transformation of normal myeloid cells into leukemic cells put forth by LeBeau et al[30] seems to be unlikely. These findings are not consistent with our previous observation of a major deletion of the p53 gene in the HL-60 promyelocytic cell line.[27] We would like to speculate that the rearrangement of the p53 gene in HL-60 cells was caused by a change in the karyotype involving the loss of chromosome 17 during the continuous passage of these cells in culture. Alternatively, it is possible that APL patients exhibit at a low frequency an additional rearrangement occurring in the short arm of chromosome 17. Therefore, the rearrangement in one APL patient as well as the deletion in the p53 gene in HL-60 cells may be an authentic observation.

In summary, the p53 protein is expressed in B-type lymphoproliferative diseases and in the majority of ALL patients as well as in lymphoid cell lines of both T and B origin, including Nalm-1 cells. In these malignancies, p53 may provide a novel tool for monitoring the activity of malignancies. Conversely, p53 is not expressed in AML, myeloproliferative diseases, and myeloid leukemic cell lines. Understanding of these variations in p53 expression in the different tumor cells may provide some insight into the biologic roles of p53 in normal and malignant cells.

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Marrow Transplantation for Severe Aplastic Anemia: Methotrexate Alone Compared With a Combination of Methotrexate and Cyclosporine for Prevention of Acute Graft-Versus-Host Disease

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Forty-six patients with severe aplastic anemia (median age, 23 years) were treated with high-dose cyclophosphamide followed by infusion of marrow from an HLA-identical family member. To evaluate postgrafting prophylaxis for graft-*v*-host disease (GVHD), they were entered into a prospective randomized trial comparing the effect of a combination of methotrexate and cyclosporine (n = 22) to that of methotrexate alone (n = 24). Forty-four of the forty-six patients had evidence of sustained marrow engraftment. Only one patient in each of the two study groups showed graft rejection. A significant reduction in the cumulative incidence of grades II to IV acute GVHD was seen in patients given methotrexate/cyclosporine (18%) compared with those given methotrexate alone (53%) (*P* = .012). In three patients given methotrexate alone, grade III developed, and in six, grade IV acute GVHD developed, compared with none given methotrexate/cyclosporine. Eighteen of the 22 patients given methotrexate/cyclosporine and 15 of the 24 given methotrexate alone are alive between 5.5 and 44.5 months (median, 18 months), with actuarial survival rates at 2 years of 82% and 60%, respectively (*P* = .062). The incidence of fatal infections was higher in patients given methotrexate alone, whereas there are as yet no significant differences in the incidence of chronic GVHD. We conclude that methotrexate/cyclosporine treatment resulted in a significant decrease in the incidence and severity of acute GVHD in patients who received transplants for severe aplastic anemia and thus an improvement in survival.

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TRANSPLANTATION of marrow from HLA-identical siblings has been used successfully to treat patients with severe aplastic anemia.[1-6] Acute graft-*v*-host disease (GVHD) has remained a serious problem adversely affecting survival. Studies in experimental animals have shown that administration of immunosuppressive agents after grafting can be effective in obviating GVHD. Methotrexate, cyclosporine, and cyclophosphamide have been frequently used for prevention of GVHD in humans. Three randomized prospective comparisons of methotrexate and cyclosporine in patients with leukemia have shown either no or only marginal reduction in the incidence of acute GVHD in patients given cyclosporine, without significant improvement in survival.[7-9] Two recent studies in dogs given DLA-nonidentical unrelated and DLA-haploidentical littermate marrow grafts have demonstrated a significant decrease in the incidence of acute GVHD, with improvement in survival when animals were given a short course of methotrexate combined with cyclosporine compared with either methotrexate or cyclosporine alone.[10,11] We have carried out a prospective randomized trial comparing the usefulness of the combination of methotrexate and cyclosporine to that of methotrexate alone in patients with severe aplastic anemia given HLA-identical sibling marrow grafts. We report this experience with 46 patients whose cases have been followed for 5½ months to 3¾ years after transplantation.

MATERIALS AND METHODS

From Dec 14, 1981, to March 14, 1985, 46 patients were entered into the study. Patient characteristics are shown in Table 1. There were no significant differences between the two study groups as regards patient characteristics. Marrow donors and recipients were HLA-A and -B identical siblings whose cells were mutually nonreactive in mixed leukocyte culture in 45 cases, and one patient given methotrexate/cyclosporine received marrow from the phenotypically HLA-identical father. The safety of the methotrexate/cyclosporine combination was first established in a pilot study including five patients (unpublished). Until Sept 13, 1984, Food and Drug Administration regulations prohibited the use of cyclosporine in children below the age of 12 years; these patients were excluded from either arm of the current study, and methotrexate was administered to all of them.

Patients were referred after detailed consultation with their physicians and admitted after outpatient and inpatient conferences that fully outlined the advantages and disadvantages of the transplantation procedure. Protocols and consent forms were approved by the Institutional Review Board at the Fred Hutchinson Cancer Research Center.

Before transplantation, all patients received cyclophosphamide, 50 mg/kg, intravenously (IV) on each of four successive days.[1,12,13] Thirty-six hours after the last dose of cyclophosphamide, donor marrow was infused IV. The day of marrow infusion was designated day 0. All multiply transfused patients 17 years and older were also given viable donor buffy coat cells as reported previously.[14,15]

Postgrafting immunosuppression was assigned by random permutations of a set of numbers known only to the protocol registrar. Assignment was stratified by patient age (≤25, >25 years), laminar airflow isolation room and decontamination, history of prior transfusion, and results of the relative response in mixed leukocyte culture. The relative response was obtained by comparing the response to sibling cells in a mixed leukocyte culture of patient cells to their response to pooled freshly drawn cells from two unrelated donors or cryopreserved cells from three unrelated donors; results were then expressed as a ratio, the relative response.[14,16] On the basis of data on

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Table 1. Patients Studied

	Methotrexate/ Cyclosporine	Methotrexate
No. of patients studied	22	24
Age (yr)		
First quartile	14	18.5
Median	23	22.5
Third quartile	34	27.5
Sex (F/M), no. of patients	9/13	17/7
Possible causes of aplastic anemia (no. of patients)		
Unknown	18	17
Drug or chemical	2	3
Hepatitis	1	3
Other	1	1
Duration of aplastic anemia in days, median (range)	40 (9–363)	41 (8–6,821)
No. of untransfused patients	3	8
Preceding transfusions in units,* median (range)		
RBC	5 (1–12)	12 (1–32)
Platelets	12 (4–700)	40 (4–96)
Refractory to random donor platelets	5	6
History of treatment with (no. of patients)		
Antithymocyte globulin	2	1
Androgens	3	7
Prednisone	8	11
No. of patients in laminar airflow room	16	18
Donor		
Age in years, median (range)	19.5 (5–40)	24 (11–41)
Sex, F/M (no. of patients)	11/11	13/11
Median (range) number of marrow cells infused (× 10^{-8}/kg)	2.9 (0.7–6.7)	2.5 (0.4–5.0)
Median (range) number of buffy coat cells infused in transfused patients (× 10^{-8}/kg)	0.9 (0.4–1.4)	0.7 (0.2–2.7)

*In many cases, exact information on the transfusion history could not be obtained; the patients' records merely indicated that multiple transfusions had been administered.

212 healthy HLA-identical siblings, a positive relative response was defined as ≥2.6% and a negative one as <2.6%. The average relative response in this control group was 0.0%, with 2 SD = 2.6%. A positive relative response was interpreted to be an expression of transfusion-induced sensitization of the patient against the marrow donor. Only three patients given methotrexate/cyclosporine and one given methotrexate alone had a positive relative response of more than 2.6%[14,16]; in one patient given methotrexate, the relative response was not evaluable.

Patients randomized to receive methotrexate/cyclosporine were given methotrexate, 15 mg/m^2 IV on day 1 and 10 mg/m^2 on days 3, 6, and 11 postgrafting.[1] Reductions or omissions of methotrexate doses were common and were due to renal insufficiency, marrow toxicity, or liver function abnormalities associated with severe GVHD. Cyclosporine treatment was begun on the day before transplantation and was given IV at 1.5 mg/kg every 12 hours until recovery from chemotherapy-induced gastrointestinal toxicity.[7-9] At that time, patients were given cyclosporine orally at 6.25 mg/kg every 12 hours. The full dose of cyclosporine was given until day 50 unless nephrotoxicity developed; the dose was reduced by 50% if the serum creatinine level doubled above baseline values and was temporarily withheld if the creatinine value exceeded 2 mg/dL. After day 50 cyclosporine therapy was decreased by 5% per week and stopped 6 months after transplantation. Serum samples for the determination of cyclosporine levels were obtained three times weekly, 12 hours after the last cyclosporine dose. Samples were analyzed by radioimmunoassay.[17]

Patients randomized to receive methotrexate alone were given methotrexate, 15 mg/m^2, IV on day 1 and 10 mg/m^2 on days 3, 6, 11, and 18 and once weekly thereafter until day 102.[1] The methotrexate dose was reduced or occasionally held in case of renal impairment.

Sixteen patients given methotrexate/cyclosporine and 18 given methotrexate were treated in laminar airflow isolation rooms with skin and gut decontamination[18,19]; assignment to these rooms depended on availability. The remainder were treated in conventional reverse-isolation rooms.

Documentation of hematopoietic engraftment and assessment and grading and treatment of acute and chronic GVHD were done as previously described.[1,14,20] Primary therapy of established grades II to IV acute GVHD consisted of methylprednisolone, 2 mg/kg/d, either IV or orally in divided doses for a period of seven to 14 days when the patient's status was reevaluated.[21] Progressive GVHD was treated with antithymocyte globulin or, in the case of methotrexate-treated patients, with cyclosporine.[21-23] Chronic GVHD was treated with methylprednisolone, either alone or combined with azathioprine.[24] Routine marrow aspirates and cytogenetic studies were usually performed on days 14, 21, 28, 56, 84, and 365 postgrafting to assess the quality of engraftment.

Results of the study were analyzed as of Aug 31, 1985. The primary factor analyzed was the occurrence of grades II to IV acute GVHD. The summary analyses of the trial used Cox's relative risk regression model.[25] Kaplan-Meier estimates of the incidence of acute GVHD and of the survival curves for the two treatment arms were also calculated.[26]

RESULTS

Table 2 summarizes the postgrafting results.

Engraftment, transfusion support, early toxicities, and graft rejection. All 46 patients showed initial evidence of hematopoietic engraftment as determined by rising peripheral blood counts, marrow cellularity, and blood genetic marker studies. The speed of recovery of granulocyte counts and the time to discontinuation of platelet and RBC transfusion support were identical in the two groups, as was the incidence of oral mucositis. There was a significantly higher incidence of elevated bilirubin levels during the first 14 days after grafting in patients given methotrexate/cyclosporine compared with methotrexate alone. Renal function impairment was a frequent complication in patients given methotrexate/cyclosporine. Hypertension (diastolics ≥90 mm Hg on at least two separate daily measurements) was seen in nine patients given methotrexate/cyclosporine and six given methotrexate; four of the six patients receiving methotrexate prophylaxis, however, were being treated with additional cyclosporine for established GVHD. There was no significant difference between the two groups in the number of days spent in the hospital.

Cyclosporine trough levels for weeks 1, 2, 3, 4, 5, 6, and 7 (means ± 1 SD) were 99 ± 74, 148 ± 116, 134 ± 126, 187 ± 207, 105 ± 103, 120 ± 94, and 130 ± 82 ng/dL, respectively. Patients received approximately 75% of the calculated cyclosporine dose. Renal function impairment or marrow toxicity led to reductions in methotrexate doses in some patients. Specifically, 13 patients given the combination of drugs received 100% of the calculated methotrexate dose, three received between 80% and 90%, and six received less than 80%. Eight of the patients given methotrexate alone received 100% of the calculated drug dose, five received between 80% and 97%, and 11 received less than 80%. A major reason for reductions or omissions of methotrexate doses in this group of patients was severe liver function abnormalities in patients with grades III and IV acute GVHD.

One patient in each group rejected the marrow graft following initial engraftment. Both patients successfully received retransplants 194 and 203 days after the initial transplant, using marrow from the same donor and following a conditioning regimen consisting of cyclophosphamide, 50 mg/kg, on each of four successive days alternating every 12 hours with antithymocyte globulin, 30 mg/kg IV, for a total of three doses.[27] One patient is surviving more than 324 days after the first graft, and one died of complications associated with chronic GVHD on day 393.

Acute GVHD. Grades II to IV acute GVHD developed in 18% of the patients given methotrexate/cyclosporine compared with 53% of those given methotrexate alone (Fig 1). No patient receiving methotrexate/cyclosporine had grades III or IV acute GVHD compared with three and six patients, respectively, given methotrexate alone (Table 2). Table 3 gives relative risk estimates from a Cox regression model. The reduction in the incidence of acute GVHD in methotrexate/cyclosporine-treated patients was significant, even when other risk factors were considered. None of the other factors studied significantly altered the rate of acute GVHD, although a suggestively higher incidence was noted in patients older than 25 years.

Chronic GVHD. There was no statistically significant difference between the two patient groups with regard to the probability of chronic GVHD developing (Table 2). Mean

Table 2. Posttransplantation Results

	Methotrexate/ Cyclosporine	Methotrexate	*P* Value
No. of patients studied	22	24	
No. of patients alive	18	15	
Median follow-up of surviving patients in months (range)	19 (10.6–44.5)	16.5 (5.5–42)	
Median day to granulocytes >1,000/μL (range)	24.5 (18–33)	25 (14–49)	.49*
Median day to discontinuation of platelet transfusions (range)	22.5 (3–118)	28 (11–191)	.25*

Table 2. Posttransplantation Results (Cont'd)

	Methotrexate/ Cyclosporine	Methotrexate	*P* Value
Median day to discontinuation of RBC transfusion (range)	53.5 (8–119)	79 (6–339)	.88*
No. patients with marrow graft rejection	1	1	
Median days in hospital (range)	40 (27–94)	45 (28–244)	.15*
Oral mucositis (no. of patients)			
None	12	14	
Mild	7	8	NS
Moderate	2	1	
Severe	1	1	
Median (range) peak bilirubin level during first 2 weeks (mg/100 mL)	2.9 (0.8–12.1)	1.2 (0.6–17.7)	.003*
Doubling of creatinine level greater than baseline or creatinine level greater than 2 mg/100 mL (no. of patients)	13	8	<.10†
Dialysis (no. of patients)	1	4	
Hypertension (no. of patients)‡	9	6§	
Causes of death (no. of patients)			
CMV IP with/without GVHD	0/0	2/0	
Idiopathic IP with/without GVHD	1/0	0/1	
Other infections with/without GVHD	1/1	6/0	
Veno-occlusive disease of liver, hemorrhage	1	0	
Total	4	9	
Acute GVHD‖ (no. of patients)			
Grade 0	16	10	
I	2	0	
II	3	4	
III	0	3	
IV	0	6	
Chronic GVHD (no. of patients)¶			
Yes	9	6	
No	10	8	.69#

Abbreviations: NS, not significant; CMV, cytomegalovirus; IP, interstitial pneumonia.

*Rank sum test statistic.

†Chi-square test.

‡Defined as diastolics ≥90 mm Hg on at least two separate daily measurements.

§Four of the six patients were being treated with additional cyclosporine for established GVHD.

‖Excludes patients who rejected their first graft.

¶In patients who lived at least 6 months after transplantation with sustained first grafts.

#Log rank statistics.

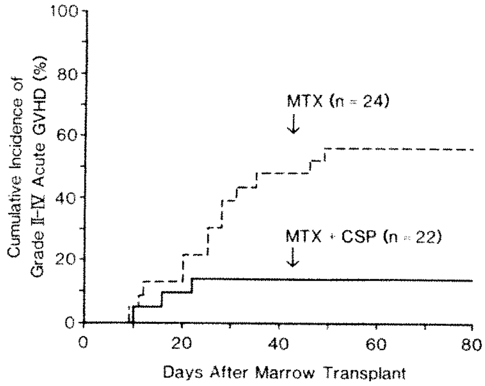

Fig 1. Probability of grades II to IV acute GVHD developing in 46 patients with severe aplastic anemia given marrow grafts from HLA-identical family members and GVHD prophylaxis with either methotrexate/cyclosporine (MTX + CSP) or methotrexate alone (MTX) (Kaplan-Meier product limit estimates[26]).

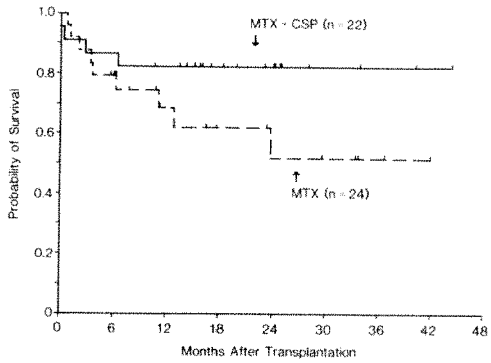

Fig 2. Probability of survival in 46 patients with severe aplastic anemia given marrow grafts from HLA-identical siblings and GVHD prophylaxis with either methotrexate/cyclosporine (MTX + CSP) or methotrexate alone (MTX) (Kaplan-Meier product limit estimates[26]). Tick marks represent surviving patients. Survival is as of Aug 31, 1985.

(median) Karnofsky performance scores among patients with chronic GVHD in the two groups are 85% (90%) and 83% (80%), respectively. All nine surviving patients with chronic GVHD in the methotrexate/cyclosporine-treated group and all six in the methotrexate-only group are presently receiving immunosuppressive treatment.[24]

Survival. The projected survival rate at 2 years is 82% for patients receiving methotrexate/cyclosporine compared with 60% for those receiving methotrexate alone (Fig 2). A Cox regression analysis (Table 3), also considering other prognostic factors, showed a suggestive survival advantage for the group receiving methotrexate/cyclosporine (P = .062). A significant beneficial influence on survival was the treatment of patients in laminar airflow room isolation. The influence of age on survival could not be assessed with this model since no patient below the age of 15 in either treatment group died.

Causes of death. Predominant causes of death were interstitial pneumonias and bacterial/fungal infections associated with GVHD (Table 2). This was most marked among patients receiving methotrexate only, with eight of the nine deaths attributable to these complications.

One patient given methotrexate died of interstitial pneumonia not associated with GVHD. He had had an unsuccessful course of immunosuppressive therapy with antithymocyte globulin before transplantation.

Among the patients given methotrexate/cyclosporine, veno-occlusive disease of the liver developed in one 43-year-old, and this patient died of hemorrhage on day 12. Another 40-year-old patient whose aplastic anemia was due to gold therapy of rheumatoid arthritis had an unsuccessful course of antithymocyte globulin therapy before transplantation. She was admitted with multiple bacterial/fungal brain and liver abscesses and died of these complications three days after transplantation.

Overall, nine of the 14 deaths in the two patient groups were related to complications from acute GVHD. Two additional patients died of problems associated with chronic GVHD.

Table 3. Cox Regression Analysis of Data on Acute GVHD and Survival

Factor	Relative Risk Estimates*	
	Grade II-IV Acute GVHD	Death
Preceding transfusions	2.76 (.131)	3.26 (.120)
Laminar airflow isolation	0.83 (.743)	0.12 (<.001)
Age 15-25 years	5.51 (.121)	
Age >25 years	6.76 (.077)	
Methotrexate/cyclosporine	0.18 (.012)	0.32 (.062)

*In parentheses are P values for testing the hypothesis that relative risk = 1.

DISCUSSION

Analogous with results in marrow-transplanted dogs,[28] three prospective clinical trials in patients with leukemia comparing methotrexate and cyclosporine treatment as single agents have shown either no or only marginal decreases in the incidence of acute GVHD in cyclosporine-treated patients without improving survival.[7-9] Subsequent experimental studies showed an impressive reduction of GVHD in dogs given a combination of methotrexate and cyclosporine. The survival rate of dogs given DLA-nonidentical unrelated marrow grafts was increased from 6% with either drug alone to 35% with the combination,[10] and that of recipients of DLA-haploidentical littermate marrow from 8% to 70%.[11] The current clinical trial was based on these experimental data. It showed methotrexate/cyclosporine therapy to be superior to methotrexate alone in preventing acute GVHD, thereby reducing the frequency of fatal infections and improving survival of patients given marrow grafts for severe aplastic anemia. Adjusting for age, the survival of patients given methotrexate alone was comparable to that reported previously.[4] FDA regulations prohibited the use of cyclosporine in patients below the age of 12 years for most of the current trial; historically, these young patients have a lower incidence of acute GVHD and better survival than older patients. The six patients excluded from this study because of young age were given methotrexate as GVHD prophylaxis, and all are surviving.

The long-term success of marrow grafting for the treatment of severe aplastic anemia depends on avoiding all of three mutually related complications: graft failure/graft rejection, GVHD, and severe infections. Any programs aimed at GVHD prevention must take the interrelationships between these three problems into account. It is conceivable that some graft-*v*-host reaction is required to eradicate residual host cells that survive the conditioning regimen. Such a possibility is in line with the clinical observation of a significant reduction in the incidence of graft rejection in patients given viable peripheral blood buffy coat cell infusions from the marrow donor.[14] Despite a significant decrease in the incidence and severity of GVHD in present patients given methotrexate/cyclosporine, graft failure was not a problem. In fact, only one patient in each study group rejected, although 35 of the 46 patients had had preceding transfusions. The reasons for the decrease in the incidence of rejection over previously reported figures are conjectural but may include lessened sensitization to non-HLA antigens through the use of buffy coat "free" red cell transfusions, single-donor platelet transfusions, and avoidance of family member transfusions before transplantation. Another indication for a lessened incidence of sensitization to blood products is the finding that only four of the current 35 transfused patients had a positive relative response in mixed leukocyte culture. This compares with the 20% to 30% reported by us previously.[16,29,30] These observations suggest that any program aimed at decreasing the incidence of rejection in transfused patients with aplastic anemia must take these changing conditions into account and emphasize the importance of randomized prospective studies. Other transplant teams have also observed reductions in the incidence of graft rejection, and this has been ascribed to the use of cyclosporine postgrafting, or total-lymphoid, total-body, or thoracoabdominal irradiation in the conditioning regimens.[3,5,6,31-34]

Patients with GVHD generally do not die of target organ failure, but rather of associated infectious problems. Effective GVHD prevention should lead to a reduction in the incidence of and mortality from infections, including interstitial pneumonias. This is evident in the current study where the number of deaths not associated with GVHD was comparable between the two treatment groups, whereas the number of deaths associated with GVHD and infections was fourfold greater among patients given methotrexate only. Reductions in the incidence of infections were seen by some but not all investigators in patients with leukemia in whom GVHD was avoided by the use of T cell–depleted marrow grafts.[35-37] A study from Minnesota showed a significant reduction in the incidence of acute GVHD with a combination of methotrexate, antithymocyte globulin, and prednisone compared with methotrexate alone, with no resultant benefit in survival, presumably because mortality from infections was not reduced.[38]

Although the incidence of acute GVHD and associated complications has been reduced and early survival improved in patients given methotrexate/cyclosporine, the incidence of chronic GVHD has been identical in the two treatment groups. Clearly, the combination of methotrexate and cyclosporine has not eliminated this problem. The lack of a difference between the two groups may in part be explained by the fact that many of those patients given methotrexate alone who would have been at highest risk for chronic GVHD developing[39] have died early of complications of acute GVHD, whereas comparable patients in the methotrexate/cyclosporine-treated group survived long enough to be at risk for chronic GVHD.

The use of laminar airflow room isolation, previously shown to reduce significantly the overall incidence of GVHD and increase survival,[40] had a less pronounced effect on the incidence of acute GVHD in the present study while continuing to show a significant beneficial effect on long-term survival. The effectiveness of methotrexate/cyclosporine in reducing the incidence and severity of acute GVHD may have in part obscured the beneficial influence of laminar airflow room isolation.

A major concern with regard to the use of any postgrafting immunosuppression is drug toxicity. The speed of recovery of blood counts and the incidence and severity of oral mucositis were similar in the two groups of patients; however, patients in the methotrexate/cyclosporine-treated group showed a higher incidence of transient liver function abnormalities, predominantly bilirubin elevations during the first 14 days. Presumably this was the result of an additive or synergistic toxic effect of methotrexate/cyclosporine combined with the toxicity of the conditioning regimen. Indeed, one patient in the methotrexate/cyclosporine group, aged 43 years, died of complications associated with severe veno-occlusive disease of the liver, presumably the result of drug toxicity. Other previously reported cyclosporine toxicities such as nephrotoxicity[41] and hypertension[42] were frequent findings in the present study. Nephrotoxicity is a particular problem during the early postgrafting period, potentiated by the use of nephrotoxic antibiotics including amphotericin B.[43] The early renal function abnormalities required reduction or omission of methotrexate and cyclosporine doses in some patients. The impact of drug dose reductions or omissions on subsequent development of acute GVHD is difficult to evaluate. The fact that more patients given methotrexate required dialysis was probably related to the fact that these patients had severe acute GVHD for which they were given additional treatment with cyclosporine.

We conclude that the combination of methotrexate and cyclosporine resulted in a significant decrease in the incidence and severity of acute GVHD in patients who received transplants for severe aplastic anemia, with a resultant improvement in survival. However, the latter conclusion is only tentative since follow-up of these patients is still short, with the longest survivor at 4 years.

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Enhancement of the Proliferation of Human Marrow Erythroid (BFU-E) Progenitor Cells by Prostaglandin E Requires the Participation of OKT8-Positive T Lymphocytes and Is Associated With the Density Expression of Major Histocompatibility Complex Class II Antigens on BFU-E

By Li Lu, Louis M. Pelus, Hal E. Broxmeyer, Malcolm A.S. Moore, Maria Wachter, Donald Walker, and Erich Platzer

The relationship between major histocompatibility complex class II antigens (MHC class II, eg, HLA-DR, Ia), T lymphocytes, and the enhancement of erythroid colony formation from BFU-E by prostaglandin E was analyzed using normal bone marrow cells. In primary methylcellulose culture, the addition of prostaglandin E_1 (PGE_1) to unseparated buffy coat, low-density, or nonadherent low-density (NAL) marrow cells resulted in an enhancement of the total number of erythroid (BFU-E) colonies observed. Treatment of bone marrow cells with a monoclonal antihuman MHC class II antibody plus complement (C′) resulted in a reduction of the total number of colonies by approximately 50% and abrogation of the enhancing effect of PGE_1. Analysis of accessory cell requirements by depletion of both adherent cells and sheep erythrocyte rosetting lymphocytes (E^+ cells) and reconstitution using C′ or anti-MHC class II antibody plus C′-treated T cell-depleted NAL ($NALT^-$) marrow cells and E^+ cell populations treated with C′ or anti-MHC class II antibody plus C′ demonstrated a requirement for MHC class II antigen$^-$ T cells, but not adherent cells, and a requirement for MHC class II antigen+ BFU-E in order to observe the enhancing effect of PGE_1 on erythroid colony formation. Positive selection of BFU-E in $NALT^-$ bone marrow expressing differing density distributions of MHC class II antigens was accomplished with monoclonal anti-MHC class II antibodies and sorting with a fluorescence-activated cell sorter (FACS). Addition of E^+ cells to the different populations of MHC class II antigen$^+$ $NALT^-$ cells demonstrated that the PGE-enhancing effects on erythroid colony formation were directly related to increasing density distributions of MHC class II antigens on BFU-E. Colony formation by BFU-E expressing a low density distribution of MHC class II antigens or having no detectable MHC class II antigens, as determined by FACS analysis, was not enhanced by PGE_1 in the presence of MHC class II antigen-positive or -negative T cells.

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A RELATIONSHIP between expression of major histocompatibility complex (MHC) class II antigens (eg, HLA-DR, Ia) on human bone marrow multipotential (CFU-GEMM), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) progenitor cells during DNA synthesis and the regulatory effects of prostaglandin E (PGE) and acidic isoferritins on the proliferation of these cells in vitro has been reported.[1-5] We[6,7] as well as others[8] have shown that PGE has the capacity to augment the number of human marrow BFU-E colonies that can be detected in vitro. In addition, we have implicated MHC class II antigens in the erythroid-enhancing effect of PGE on BFU-E.[6]

Considerable evidence has accumulated indicating that either T lymphocytes[9-12] or monocytes-macrophages,[8,13-17] both cell populations,[18-21] or other cells[22] play roles in the regulation of BFU-E proliferation. In this report, we have investigated the role of accessory cells and MHC class II antigens on the enhancement of bone marrow BFU-E proliferation by PGE_1. Evidence is presented that indicates that PGE_1 specifically enhances colony formation by BFU-E expressing a high density distribution of MHC class II antigens and that this effect requires the participation of a population of $OKT8^+$ lymphocytes that do not have to express MHC class II antigens.

MATERIALS AND METHODS

Cells and cell separation procedures. Bone marrow cells were obtained by aspiration from the posterior iliac crest of healthy volunteers who had given informed consent. Unseparated nucleated buffy coat cells were obtained by centrifugation of whole marrow aspirates at 1,500 rpm for 10 minutes and aspiration of the white cells that overlay the red cell layer. Low-density cells (LD, <1.077 g/mL) were obtained after separation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) at 1,500 rpm for 30 minutes, washed three times, and resuspended in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS, Hyclone, Sterile Systems, Inc, Logan, Utah). Cells were further separated into nonadherent and adherent cells after incubation on plastic tissue culture dishes (Falcon 3003, Falcon Plastics, Division of Becton Dickinson, Rutherford, NJ) for 90 minutes at 37 °C in 5% CO_2. Nonadherent cells were collected by gently swirling the dishes and slowly removing the suspension cells. Nonadherent cells prepared in this manner routinely contained $<2\%$ naphthyl-acetate-esterase-positive cells. Adherent cells were obtained using a rubber policeman after washing the dishes five times. The adherent cell fraction contained 90% to 98% monocytes/macrophages as determined by naphthyl-acetate-esterase staining.

Nonadherent low-density cells (NAL) were further separated into E rosette-positive (E^+) and -negative (E^-) populations according to a method modified from Weiner et al.[23] Briefly, sheep erythrocytes

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(SRBC) were washed three times in Ca^{++}-free and Mg^{++}-free phosphate-buffered saline (PBS), resuspended at 5% (vol/vol) in medium, and treated with neuraminidase (Calbiochem-Behring Corp, La Jolla, Calif) for 30 minutes at 37 °C. After two washes, 1 mL of 5% neuraminidase-treated SRBC was mixed with 10 mL of NAL marrow cells (4×10^6 cells/mL), incubated for five to ten minutes at 37 °C, centrifuged at 1,000 rpm for five minutes, and allowed to stand for 90 minutes at 4 °C. The pellet was then resuspended gently, placed on Ficoll-Hypaque, and centrifuged at 2,000 rpm for 20 minutes. Interface cells were washed three times and constituted the T cell–depleted NAL (NALT$^-$) fraction. Rosette-forming cells recovered in the pellet were treated with Tris–ammonium chloride to lyse the SRBC, washed three times, and constituted the E+ fraction. The E+ fraction contained approximately 95% T cells as determined using the OKT11a (anti-SRBC) pan–T cell antibody. The NALT$^-$ marrow cell fraction usually contained about 5% OKT11a$^+$ cells and were treated with OKT11a and complement (C′) to remove remaining T lymphocytes.

Analysis of T lymphocyte subsets. The purity of the T cell populations was analyzed by indirect immunofluorescence using a FACS IV (Becton Dickinson, Sunnyvale, Calif). One million cells were incubated with 1:50 dilutions of monoclonal anti–T lymphocyte antibodies for 40 minutes at 4 °C, washed three times, and then incubated with a 1:40 dilution of affinity-purified goat antimouse IgG F(ab′)$_2$ fluorescein isothiocyanate (FITC) (Cappel Laboratories, Cochranville, Pa) for 40 minutes at 4 °C. After washing three times, cells were brought up to 1 mL with PBS for fluorescein-activated cell sorter (FACS) analysis. Cells were maintained at 4 °C until analyzed.

Positive selection of T lymphocyte subsets. E$^+$ cells were sorted into monoclonal antibody–positive and –negative populations using a FACS IV or FACS 440. Ten million cells were treated in 0.5 mL of medium containing OKT4 or OKT8 antibodies at a final dilution of 1:200 for 40 minutes at 4 °C. Following incubation, the cells were washed three times and treated with FITC-labeled goat antimouse IgG (heavy + light, F(ab′)$_2$) for 40 minutes at 4 °C. The cells were subsequently washed three times and resuspended in PBS with 1% FCS for sterile sorting. The gating criteria on the FACS were set for high (positive) and low (negative) fluorescence intensity windows separated by an intermediate zone of 40 channels containing approximately 40% of the sorted cells, which were discarded.[24] After sorting, the recovered high- and low-fluorescent intensity cell populations were washed twice and resuspended in media containing 10% FCS. The viability of sorted cells was greater than 95% using trypan blue. The purity of sorted cells was analyzed immediately by indirect immunofluorescence using a FACS. All monoclonal antibodies were dialyzed to remove contaminating sodium azide prior to being used for cell sorting.

Positive selection of NALT$^-$ marrow cells expressing different density distributions of MHC class II antigens. NALT$^-$ cells were sorted with a FACS using monoclonal anti-MHC class II antibody (1:200 dilution) into populations containing high (Ia^{+++}), medium (Ia^{++}), low (Ia$^+$), or nondetectable (Ia$^-$) density distributions of MHC class II antigens as previously described for sorting of T cell subsets. Since only two windows can be collected at a time and we wished to achieve the best separation of cells based on antigen density, cells were sorted at one time for Ia^{+++} and Ia$^+$ or for Ia^{++} and "Ia$^-$" densities. The other fractions were not used. After sorting, the cells were washed and plated. Analysis of these populations was done after sorting.

Antibody and C′-dependent cytotoxicity test. All antibodies were used at specific final dilutions, predetermined for optimal activity, on cells suspended at a density of one million cells per 0.1 mL. Rabbit C′ (Low Tox-H) was purchased from Accurate Chemical Co, Westbury, NY, and used at a 1:8 dilution. Antibody plus C′ cytotoxicity reactions were carried out as described previously.[1-4] Cells were washed twice and brought back to their original starting volume prior to plating.

Assay. Bone marrow cells were plated at the concentration listed in the table legends, or what was left after antibody and C′ treatment, in 35-mm Lux standard tissue culture dishes containing a 1-mL mixture of IMDM, 1% methylcellulose, 30% FCS, 5% medium conditioned by leukocytes (LCM) from patients with hemochromatosis in the presence of 1% phytohemagglutin (PHA-LCM) and 5×10^{-5}mol/L 2-mercaptoethanol. Hemin was added in the dish at a concentration of 0.1 mmol/L when LD, NAL, or NALT$^-$ cells were used to increase the cloning efficiency of BFU-E and CFU-GEMM.[7,25] Cells were plated with or without PGE$_1$. One unit of a Step III preparation of sheep plasma erythropoietin (Connaught Laboratories, Ltd, Willowdale, Ontario, Canada or Hyclone, Sterile Systems, Logan, Utah) was added to the dish on day 0. Indomethacin was used at a final concentration of 10^{-6}mol/L to prevent endogenous prostaglandin production.[3] Dishes were incubated at 37 °C in a humidified atmosphere flushed with 5% CO$_2$ in air. Colonies were scored after 14 days of incubation.

Reagents. The monoclonal antihuman MHC class II antibody NEI-011 was purchased from New England Nuclear, Boston. Monoclonal antihuman T cell antibodies OKT3, OKT4, and OKT8 were purchased from Ortho Pharmaceutical Corp, Raritan, NJ. PGE$_1$ was used at a final concentration of 10^{-7}mol/L. Indomethacin was used at a final concentration of 10^{-6}mol/L. Both PGE$_1$ and indomethacin were purchased from Sigma Chemical Co, St Louis. Bovine hemin was purchased from Eastman Kodak Co, Rochester, NY, and was prepared in 0.5 mol/L NaOH and then neutralized with 1 N HCl as described.[25]

Statistical analysis. Levels of significance for comparisons between samples were determined using Student's *t* distribution. The results are expressed as the mean ± 1 SEM of four or five plates per point for each experiment.

RESULTS

Influence of accessory cell populations on the enhancement of erythroid colony formation from BFU-E by PGE$_1$. The addition of 10^{-7}mol/L PGE$_1$ to cultures containing BFU-E in unseparated (buffy coat), LD, or NAL bone marrow cells resulted in a significant enhancement of erythroid colony formation from BFU-E (67 ± 17, 64 ± 11, and 48 ± 9%, respectively, average for three experiments) in comparison with control cultures (Table 1). In all cases, treatment of these marrow cell populations with a monoclonal anti-MHC (α-MHC) class II antibody plus C′ decreased the total number of erythroid colonies detected by 47% to 58% and abrogated the enhancing effect of PGE$_1$ on colony formation. Removal of T lymphocytes from NAL marrow cells by rosetting with sheep erythrocytes resulted in the inability to detect PGE$_1$ enhancement of erythroid colony formation from BFU-E, suggesting that T lymphocytes may be needed to observe PGE$_1$ enhancement of BFU-E proliferation. Treatment of NALT$^-$ bone marrow with α-MHC class II antibody plus C′ removed approximately 48% of BFU-E in a similar fashion to its effect on buffy coat, LD, or NAL marrow cells.

Influence of T lymphocytes and monocytes-macrophages on the enhancement of erythroid colony formation from BFU-E by PGE$_1$. The effects of T lymphocytes and monocytes-macrophages on the enhancement of BFU-E colony formation by PGE$_1$ were evaluated using cell depletion and

Table 1. Influence of Cell Populations From Normal Bone Marrow on the Enhancement of BFU-E by PGE_1

Cell Population*	Treatment	Colonies (BFU-E)† Control Media	Colonies (BFU-E)† $+10^{-7}$ mol/L PGE_1	Colonies (BFU-E)† Percent Change From Control	Percent Change From Control‡
Buffy coat	C′	38 ± 2	74 ± 3	+94‖	+67 ± 17¶
Buffy coat	α-MHC class II + C′	20 ± 2 [−47]§	17 ± 1	−13	−3 ± 5
LD	C′	32 ± 2	49 ± 6	+53¶	+64 ± 11¶
LD	α-MHC class II + C′	17 ± 2 [−47]	18 ± 1	+6	−4 ± 5
NAL	C′	43 ± 3	59 ± 3	+37¶	+48 ± 9‖
NAL	α-MHC class II + C′	18 ± 1 [−58]	17 ± 1	−7	−3 ± 7
NALT$^-$	C′	119 ± 2	118 ± 2	−1	−1 ± 2
NALT$^-$	α-MHC class II + C′	62 ± 4 [−48]	58 ± 5	−8	−2 ± 3

Abbreviation: α, anti.

*Cells were plated at different concentrations depending upon the cell population as follows: buffy coat cells at 2×10^5, LD at 1×10^5, NAL at 1×10^5, and NALT$^-$ at 5×10^4.

†Data from one representative experiment. Quadruplicate plates were scored for BFU-E on day 14. Results are expressed as mean colonies ± SEM, and the percent change was calculated from control media.

‡Mean ± 1 SEM of the average data from three experiments.

§Reduction in BFU-E frequency following treatment with α-MHC class II plus C′ in comparison with cells treated with C′.

‖$P < .001$.

¶$P < .05$.

reconstitution experiments (Table 2). LD bone marrow cells were depleted of E^+ and plastic adherent cells and reconstituted with various ratios of either or both cell populations. The addition of E^+ marrow cells to NALT$^-$ marrow restored the enhancing effect of PGE_1 on erythroid colony formation (range, 19% to 67%, four experiments) using E^+:NALT$^-$ ratios of 0.5:10 (5% E^+) to 4:10 (40% E^+). Maximal activity was observed at an E^+:NALT$^-$ ratio of 1:10 (10% E^+). Reconstitution of NALT$^-$ marrow cells with plastic adherent cells at ratios of 5% to 40% failed to restore the enhancing effect of PGE_1 on erythroid colonies and resulted in significant dose-dependent (30% to 84%) inhibition of the number of detectable erythroid colonies. The addition of both E^+ and adherent cells at ratios of 5% and 10% were as effective as E^+

Table 2. Influence of T Lymphocytes and Monocytes-Macrophages on Enhancement of Erythroid Colony Formation From BFU-E by PGE_1

Cell Populations*	Colonies (BFU-E)† Control Media	Colonies (BFU-E)† $+10^{-7}$ mol/L PGE	Colonies (BFU-E)† Percent Change From Control	Percent Change From Control‡
NALT$^-$	81 ± 1	83 ± 2	+2	0 ± 2
NALT$^-$				
+ E^+ (5%)	82 ± 2	138 ± 2	+69§	+45 ± 10‖
+ E^+ (10%)	83 ± 1	157 ± 4	+88§	+67 ± 8§
+ E^+ (20%)	84 ± 2	108 ± 14	+28	+38 ± 5‖
+ E^+ (40%)	80 ± 1	94 ± 18	+18	+19 ± 3¶
NALT$^-$				
+ AD (5%)	57 ± 3 (30%)¶	51 ± 2	−11	−3 ± 3
+ AD (10%)	40 ± 4 (51%)¶	44 ± 2	+10	−2 ± 5
+ AD (20%)	23 ± 2 (72%)¶	22 ± 2	−5	−1 ± 3
+ AD (40%)	13 ± 2 (84%)¶	15 ± 3	+18	+7 ± 5
NALT$^-$				
+ E^+ (5%) + AD (5%)	90 ± 6	135 ± 18	+50‖	+46 ± 19‖
+ E^+ (10%) + AD (10%)	93 ± 3	136 ± 4	+47‖	+60 ± 11§
+ E^+ (20%) + AD (20%)	86 ± 5	89 ± 4	+4	+17 ± 7¶
+ E^+ (40%) + AD (40%)	81 ± 4	80 ± 6	−0.4	+1 ± 1

*NALT$^-$ bone marrow cells were plated at 5×10^4/dish alone or reconstituted with E^+ or adherent (AD) marrow cells at the ratios indicated. E^+ and AD cells formed no colonies when plated alone.

†Data from one experiment. Quadruplicate plates were scored for BFU-E on day 14. Results are expressed as mean colonies ± SEM, and the percent change was calculated from control media.

‡Mean percent change ± SEM of the average data from four experiments.

§$P < .0001$.

‖$P < .001$.

¶Percent inhibition of NALT$^-$ cells plated in the presence of control media.

Table 3. Selective Effect of PGE$_1$ on Colony Formation From MHC Class II Antigen–Positive BFU-E

	Colonies (BFU-E)†			
Treatment*	Control Media	10^{-7} mol/L PGE	Percent Change From Control	Percent Change From Control‡
NALT$^-$ (C′)	119 ± 2	118 ± 2	−1	0 ± 1
NALT$^-$ (α-MHC class II + C′)	62 ± 4	58 ± 5	−8	−5 ± 4
NALT$^-$ (C′) + E$^+$ (C′)	119 ± 5	176 ± 2	+48§	+63 ± 7§
NALT$^-$ (C′) + E$^+$ (α-MHC class II + C′)	119 ± 2	180 ± 12	+50§	+59 ± 10§
NALT$^-$ (α-MHC class II + C′) + E$^+$ (C′)	60 ± 1	64 ± 2	+8	+1 ± 3
NALT$^-$ (α-MHC class II + C′) + E$^+$ (α-MHC class II + C′)	58 ± 2	61 ± 4	+5	+5 ± 1

*NALT$^-$ bone marrow cells were plated at 5×10^4 cells/dish following treatment with C′ or monoclonal α-MHC class II antibody (1:100) plus C′. E$^+$ represents T lymphocytes added to NALT$^-$ marrow cells at 10% (5×10^3 cells) after treatment with C′ or α-MHC class II + C′. E$^+$ cells did not form colonies when tested alone.

†Data from one experiment. Quadruplicate plates were scored for BFU-E on day 14. Results are expressed as mean colonies ± SEM and the percent change was calculated from control media.

‡Mean ± 1 SEM of the mean average data from five experiments.

§$P < .005$.

cells alone in restoring the erythroid-enhancing effect of PGE$_1$. However, at ratios of 20% and 40%, they were less effective than E$^+$ cells alone, perhaps because of the inhibitory influences of the adherent cells. Neither E$^+$ nor adherent cells alone were capable of erythroid colony formation in the presence of erythropoietin and hemin (data not shown). The purity of E$^+$ cells was about 95% as determined by immunofluorescence using the monoclonal anti-SRBC receptor antibody OKT11a. The purity of adherent cells was >95% using napthyl-acetate-esterase staining.

The effect of PGE$_1$ on erythroid colony formation by MHC class II antigen–positive BUF-E. To define an accessory T lymphocyte requirement for PGE$_1$-mediated enhancement of erythroid colony formation and to identify the MHC class II antigen–positive participating cell population(s), NAL marrow cells were depleted of T lymphocytes, treated with or without α-MHC class II plus C′, and reconstituted with untreated E$^+$ cells or E$^+$ cells treated with MHC class II plus C′ prior to their addition to NALT$^-$ marrow cells (Table 3). As observed previously, removal of T lymphocytes from NAL marrow by rosetting with sheep erythrocytes resulted in the inability to observe any enhancing effect of PGE$_1$ on erythroid colony formation. Furthermore, treatment of NALT$^-$ marrow cells with MHC class II plus C′ resulted in approximately a 48% reduction in the number of detectable BFU-E. Reconstitution of NALT$^-$ bone marrow cells with E$^+$ marrow cells at a ratio of 1:10 (E$^+$:NALT$^-$) resulted in the restoration of the enhancing effect of PGE$_1$ on erythroid colony formation from BFU-E (63% ± 7%, five experiments). Treatment of E$^+$ cells with MHC class II antibody plus C′, which removed MHC class II$^+$ E$^+$ cells, prior to their addition to NALT$^-$ marrow did not affect the ability of these cells to restore the enhancing effect of PGE$_1$ on erythroid colony formation (59% ± 10%, five experiments). In contrast, treatment of NALT$^-$ marrow cells with MHC class II antibody plus C′ reduced the number of erythroid colonies detected and blocked the enhancing effect of PGE$_1$ on erythroid colony formation following reconstitution with either untreated E$^+$ cells or MHC class II antibody plus C′-treated E$^+$ cells.

The influence of the density distribution of MHC class II antigens on NALT$^-$ cells on the enhancement of erythroid colony formation by PGE$_1$ in the presence of added T lymphocytes. To evaluate further the relationship between expression of MHC class II antigens on BFU-E and the enhancing effect of PGE$_1$ on erythroid colony formation, NALT$^-$ cells were separated by FACS into fractions with Ia^{+++}, Ia^{++}, Ia$^+$, and "Ia$^-$" density distributions of MHC class II antigens. A representative presorting pattern of antigen-positive cells is shown in Fig 1, and a representative postsorting pattern of antigen-positive cells with differing fluorescent intensities is shown in Fig 2. In this experiment,

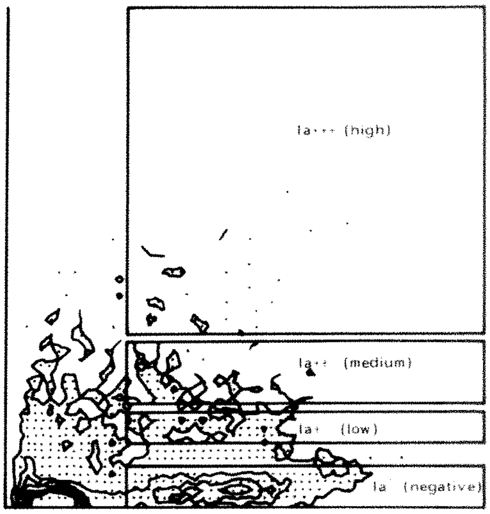

Fig 1. Representative analysis on the FACS 440 of the density distribution of MHC class II (Ia) antigens on NALT$^-$-bone marrow cells using a monoclonal antihuman MHC class II antibody (NE1-011) before sorting (x axis, forward light scatter; y axis, fluorescence intensity). Gating windows were set as follows: channels 0 to 20 contained 32% of the cells and were designated "Ia$^-$", channels 32 to 48 contained 4.8% of the cells and were designated Ia$^+$, channels 52 to 84 contained 5% of the cells and were designated Ia^{++}, and channels 88 to 255 contained 6.6% of the cells and were designated Ia^{+++}.

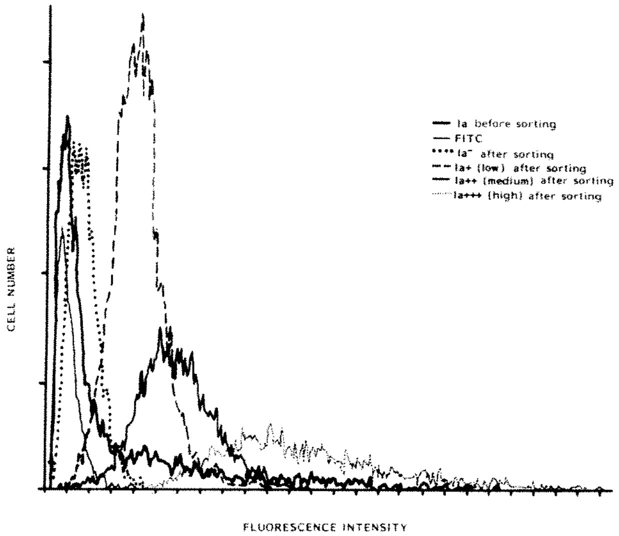

Fig 2. Profile of the density distribution of MHC class II antigen–positive cells after cell sorting on the FACS 440. The "Ia$^-$", Ia$^+$, Ia^{++} and Ia^{+++} cell fractions contained respectively at least 4%, 80%, 90%, and 92% of the cells staining for MHC class II antigens.

the "Ia$^-$", Ia$^+$, Ia^{++}, and Ia^{+++} cell fractions contained respectively at least 4%, 80%, 90%, and 92% of the cells staining for MHC class II antigens. In some experiments the "Ia$^-$" fraction contained <2% MHC class II antigen–positive cells, and the MHC class II antigen–positive fractions were composed of >95% MHC class II antigen–positive cells. The actual colony numbers for one representative experiment and the average percent change from control for three experiments using the sorted populations of NALT$^-$ cells differing in density distributions of MHC class II antigens are shown in Table 4. Because of the overlap of the Ia^{++} population with the Ia$^+$ and Ia^{+++} cells, only the "Ia$^-$", Ia$^+$, and Ia^{+++} cells were evaluated. In the presence of E$^+$ cells, PGE$_1$ enhanced erythroid colony formation by Ia$^+$ NALT$^-$ and Ia^{+++} NALT$^-$ cells, but did not enhance colony formation by the "Ia$^-$" NALT$^-$ cells. The enhancement in colony formation was greater for the cells expressing a higher density distribution of MHC class II antigens (Ia^{+++}) than for the cells expressing a lower density distribution of these antigens (Ia$^+$) and was respectively +154% ± 43% and +43% ± 10% for the two responding cell populations. The greater enhancement in erythroid colony formation using the Ia^{+++} population of cells was not due to a lower control number of Ia^{+++} *v* Ia$^+$ cells since in some of the experiments the control number of Ia^{+++} cells was higher than the control number of Ia$^+$ cells. Results were similar whether the MHC class II antigen$^+$ NALT$^-$ cells were cultured with E$^+$ cells pretreated with control medium, C′, or MHC class II plus C′ (data not shown).

Influence of T lymphocytes and their subsets on erythroid colony enhancement by PGE$_1$. The effects of treatment of bone marrow cells with monoclonal anti–T cell antibodies in the presence or absence of C′ on the enhancement of colony formation from BFU-E by PGE$_1$ were analyzed (Table 5). Treatment of unseparated bone marrow cells with either the pan-T cell antibody (OKT3 (1:50 dilution) or the T subset-specific OKT8 (1:50 dilution) antibody in the presence of C′ resulted in the inability to detect PGE$_1$-mediated enhancement of erythroid colony formation from BFU-E (2% ± 6%, 6% ± 2% enhancement, respectively, three experiments). In contrast, treatment of marrow cells with the T subset-specific OKT4 (1:50 dilution) antibody plus C′ was without effect on PGE$_1$-mediated erythroid enhancement (31% ± 4% enhancement, three experiments). In the absence of C′, pulse exposure of marrow cells for 30 minutes with OKT8 antibody was able to block the enhancing effect of PGE$_1$ on erythroid colony formation (18% ± 4% enhancement, three experiments), whereas OKT3 and OKT4 were without effect (59% ± 24%, 64% ± 12% enhancement, three experiments).

Table 4. Relationship Between the Density Distribution of MHC Class II Antigens on BFU-E and Enhancement of Erythroid Colony Formation by PGE$_1$ in the Presence of T Lymphocytes

	Colonies (BFU-E)†			
Cells*	Control Media	10^{-7} mol/L PGE$_1$	Percent Change From Control	Percent Change From Control‡
NALT$^-$ alone	63 ± 3	61 ± 3	−3	−7 ± 3
NALT$^-$ + E$^+$	60 ± 4	89 ± 2	+48§	+55 ± 7§
NALT$^-$ (Ia^{+++}) alone	16 ± 4	18 ± 2	+13	+13 ± 3
NALT$^-$ (Ia^{+++}) + E$^+$	15 ± 3	33 ± 4	+120‖	+154 ± 43
NALT$^-$ (Ia$^+$) alone	59 ± 3	60 ± 3	+2	+3 ± 2
NALT$^-$ (Ia$^+$) + E$^+$	65 ± 1	100 ± 2	+54§	+43 ± 10
NALT$^-$ ("Ia$^-$") alone	64 ± 5	62 ± 4	−3	−11 ± 6
NALT$^-$ ("Ia$^-$") + E$^+$	62 ± 7	74 ± 1	+19	−7 ± 8

*NALT$^-$ bone marrow cells were plated at 3 × 10^4 cells/dish. NALT$^-$ cells were sorted into Ia antigen high-density (NALT$^-$ Ia^{+++}), low-density (NALT$^-$ Ia$^+$) and "negative" (NALT$^-$ "Ia$^-$") fractions and plated at 2.5 × 10^4, 3 × 10^4, and 3 × 10^4 cells/dish.

†Data from one representative experiment. Results are expressed as mean colonies ± SEM and the percent change was calculated from control media.

‡Mean percent change from control medium ± 1 SEM of the mean of the average data from four experiments.

§$P < .01$.

‖$P < .001$.

Table 5. Influence of T Lymphocytes and Their Subpopulations on Enhancement of Erythroid Colonies by PGE_1

	Colonies (BFU-E)*			
Treatment	Media	$+10^{-7}$ mol/L PGE_1	Percent Change From Control	Percent Change From Control†
C′	71 ± 3	115 ± 8	+62‡	+68 ± 13‡
OKT3 + C′	60 ± 3	67 ± 4	+12	+2 ± 6
OKT4 + C′	65 ± 5	84 ± 11	+30§	+31 ± 4§
OKT8 + C′	65 ± 3	70 ± 2	+8	+6 ± 2
Media	74 ± 9	120 ± 3	+62‡	+74 ± 9‖
OKT3	76 ± 3	90 ± 3	+43§	+57 ± 24§
OKT4	74 ± 4	105 ± 10	+42§	+64 ± 12‡
OKT8	75 ± 5	84 ± 7	+11	+18 ± 4

*Results are expressed as mean colonies ± SEM per 2×10^5 normal bone marrow buffy coat cells from one experiment, and the percent change was calculated from media control.

†Mean percent change ± 1 SEM of the average data from three experiments.

‡$P < .001$.

§$P < .05$.

‖$P < .0001$.

In the presence of antibody plus C′ the appropriate cell populations were killed, and in the presence of antibody without C′ there was no effect on cell viability.

The effect of positively selected OKT8$^+$ T lymphocytes on the enhancing effect of PGE_1 on erythroid colony formation. The requirement for OKT8$^+$ T lymphocytes to observe PGE_1 enhancement of erythroid colony formation was further analyzed by positive selection of T lymphocyte subsets by sorting using the monoclonal OKT4 and OKT8 antibodies (1:200 dilution) and the FACS IV. The E$^+$ fraction of bone marrow cells was labeled with either OKT4 or OKT8 and sorted into positive and negative populations. Prior to sorting, the E$^+$ cell fraction contained >90% OKT11a$^+$ (SRBC$^+$) T cells with approximately >30% OKT4$^+$ and >60% OKT8$^+$ cells. After sorting, the OKT4$^+$ and OKT8$^+$ cell fractions contained ≥98% and ≥97% positive cells, respectively. The OKT4$^-$ and OKT8$^-$ cell fractions contained <2% cells positive for OKT4 and OKT8.

Sorted cells were added back to NALT$^-$ narrow cells (from the same donor) at ratios of 1%, 5%, 10%, 20%, and 40% (Table 6). The addition of OKT8$^+$ or OKT4$^-$ cell populations restored the ability of NALT$^-$ marrow cells to respond to the erythroid colony enhancing effect of PGE_1. Optimal activity was observed at T4$^-$ or T8$^+$:NALT$^-$ ratios of 1:10 (10%). The addition of OKT4$^+$ or OKT8$^-$ cell fractions to NALT$^-$ marrow failed to result in restoration of the enhancement of erythroid colony formation from BFU-E by PGE_1. Each of the sorted cell populations was incapable of colony formation by themselves (data not shown).

Table 6. Influence of Purified Positively Selected Marrow T Lymphocyte Subpopulations on the Enhancement of Erythroid Colony Formation From BFU-E by PGE_1

Cells*	Percent Change From Media Control in the Presence of 10^{-7} mol/L PGE_1†	Number of Experiments
NALT$^-$	+1 ± 2	3
NALT$^-$ + T8$^+$ (40%)	+28	1
NALT$^-$ + T8$^+$ (20%)	+28	1
NALT$^-$ + T8$^+$ (10%)	+43 ± 2‡	3
NALT$^-$ + T8$^+$ (5%)	+44 ± 13‡	3
NALT$^-$ + T8$^+$ (1%)	+26 ± 2§	2
NALT$^-$ + T4$^-$ (40%)	+24	1
NALT$^-$ + T4$^-$ (20%)	+27	1
NALT$^-$ + T4$^-$ (10%)	+37 ± 9‡	3
NALT$^-$ + T4$^-$ (5%)	+29 ± 5‡	3
NALT$^-$ + T4$^-$ (1%)	+22 ± 5§	2
NALT$^-$ + T4$^+$ (40%)	+7	1
NALT$^-$ + T4$^+$ (20%)	+12	1
NALT$^-$ + T4$^+$ (10%)	+6 ± 4	3
NALT$^-$ + T4$^+$ (5%)	−1 ± 1	3
NALT$^-$ + T4$^+$ (1%)	−2 ± 4	2
NALT$^-$ + T8$^-$ (40%)	+16	1
NALT$^-$ + T8$^-$ (20%)	+13	1
NALT$^-$ + T8$^-$ (10%)	+1 ± 6	3
NALT$^-$ + T8$^-$ (5%)	+5 ± 5	3
NALT$^-$ + T8$^-$ (1%)	+1 ± 1	2

*Bone marrow cells were depleted of adherent and E$^+$ cells and reconstituted with positively selected (sorted by FACS) cells at the ratios indicated. The various T cell populations and NALT$^-$ cells in the absence of PHA-LCM and erythropoietin formed no BFU-E by themselves.

†Results are expressed as mean percent change ± SEM per 5×10^4 NALT$^-$ cells or NALT$^-$ cells reconstituted with T4$^+$, T4$^-$, T8$^+$ and T8$^-$ cells from three experiments. The control numbers of the colonies from NALT$^-$ cells in the absence of PGE_1 and T cells for the three experiments were 54 ± 3, 48 ± 4, and 44 ± 4.

‡$P < .001$.

§$P < .05$.

DISCUSSION

Most or all erythroid progenitor cells express MHC class II (eg, HLA-DR, Ia) antigens on their cell surface,[26–28] although there is heterogeneity of density expression of these antigens on erythroid progenitors.[28] A role for MHC class II antigens on BFU-E and the regulation of erythropoiesis in vitro[1,6,11,12] and a role for MHC class II antigens in the enhancement of erythroid colony formation from BFU-E by PGE have been suggested.[6] This latter information has now been extended to demonstrate the participation of marrow T lymphocytes having the OKT8 subset phenotype in the selective enhancing effect of PGE_1 on colony formation by BFU-E and to show that this enhancing activity is directly associated with an increasing density distribution of MHC class II antigens on BFU-E.

The removal of bone marrow T lymphocytes using (1) selection for E^+ ($SRBC^+$) cells, (2) negative selection using purified anti-T cell monoclonal antibodies in a C′-mediated cytotoxicity assay, and (3) positive selection of subsets of marrow T lymphocytes using monoclonal antibodies and FACS sorting combined with recombination experiments indicate a requirement for OKT8 antigen-positive bone marrow T lymphocytes to observe the enhancing effect of PGE_1 on erythroid colony formation. In this regard, it is of interest that $OKT8^+$ T lymphocytes are the predominant T lymphocyte population found in human bone marrow.[29] Of interest to the $OKT8^+$ cell-mediated effect is that OKT8 antibody in the absence of C′ blocks the PGE enhancement of erythroid colony formation. This suggests that the antigenic determinant recognized by OKT8 antibody may be involved in these interactions, but this remains to be determined.

Cellular actions and interactions in the control of in vitro erythropoiesis have been an area of intensive investigation. Studies have shown that the proliferation of BFU-E can be modulated, either enhanced or inhibited, by normal peripheral blood T lymphocytes and their subpopulations, monocytes and macrophages, other cells, or combinations of these cells.[9-22] In this report, we do not directly address the question of cellular involvement in the control of BFU-E proliferation, but rather we have focused on accessory cell involvement in the erythroid-enhancing effect noted with PGE_1. Our data using marrow BFU-E are consistent with an enhancing effect of bone marrow T lymphocytes mediating the PGE_1 effect and an inhibitory effect of marrow adherent cells on the proliferation of BFU-E. The inhibitory effect of marrow adherent cells on BFU-E most likely reflects their production of acidic isoferritins, which are inhibitory of BFU-E proliferation.[1] This inhibitory effect could be abrogated by use of an antiacidic isoferritin antisera (L. Lu, unpublished observation). Our populations of $NALT^-$ marrow cells were not completely devoid of T cells and/or adherent cells, which is the case with almost all the other studies describing effects of accessory cells on proliferation of BFU-E. Therefore, sufficient residual cells may have remained or may have been generated during the culture period to mediate accessory cell function, at least with respect to their requirement for BFU-E proliferation. It should also be noted that we used bone marrow BFU-E and T cells, which is in contrast to the predominant use of peripheral blood BFU-E and accessory cells by other investigators. Also, our culture conditions permit the simultaneous detection of BFU-E and multipotential CFU-GEMM colonies, which differs from the conditions used in the experiments of other investigators. Our cultures contained a source of burst-promoting activity present in PHA-LCM. We have shown elsewhere that the effects of PHA-LCM and PGE are additive on enhancing erythroid colony formation.[7] Of direct relevance to a role for monocytes in the regulation of erythroid colony formation is that monocytes produce PGE_1.[30] Thus, monocytes have the potential (1) to enhance colony formation via the release of PGE_1, which could act via the $OKT8^+$ lymphocyte pathway, and (2) to suppress colony formation via the release of inhibitory molecules such as acidic isoferritins.[1] We had purposely added indomethacin to our cultures to suppress the production of prostaglandins so that we could assess the role of exogenously added PGE_1. Our preliminary results indicate that PGE_1 stimulates the release into conditioned medium of an erythroid-enhancing activity from sorted $OKT8^+$ cells (purity >98%), which when added to $NALT^-$ marrow cells restores the erythroid-enhancing effect of PGE_1.[31] Although these results suggest the T cell derivation of an erythroid-enhancing activity, they cannot exclude an effect of this factor mediated via an additional marrow accessory cell. Further studies using more purified marrow BFU-E populations are required to elucidate this point.

In a previous report, we had presented evidence that suggested a link between MHC class II antigen expression and the enhancement of erythroid colony formation by PGE.[6] However, these studies did not discriminate between an effect upon MHC class II $antigen^+$ BFU-E or an effect mediated via MHC class II antigen-positive accessory cells. In this study, treatment of T-depleted marrow cells and positively selected T lymphocytes with a monoclonal MHC class II antibody plus C′ prior to their being recombined suggests that PGE_1 with the participation of MHC class II antigen-negative T cells selectively enhances the proliferation of MHC class II antigen-positive BFU-E. The data shown in Tables 1 and 3 using monoclonal anti-MHC class II antibody plus C′ to remove 50% of BFU-E and to remove completely the cell populations responding to the enhancing effects of PGE_1 in combination with the data shown in Table 4 evaluating the effects of PGE_1 on the MHC class II antigen-positive cells sorted by FACS are consistent with increasing responsiveness of BFU-E to this enhancing effect being associated with an increasing density distribution of MHC class II antigens on BFU-E. That we were killing only 50% of BFU-E by monoclonal anti-MHC class II plus C′ most likely reflects removal mainly of cells with a higher density distribution of MHC class II antigens. This has been noted for CFU-GM where S-phase cells express a higher density distribution of MHC class II antigens.[2,32] Even by FACS analysis using the Nel-011 antibody, a portion of BFU-E did not contain detectable levels of MHC class II antigens. This may reflect a very low density of antigens on these cells.

It is difficult to extrapolate from findings in vitro to relevance in vivo, especially when so many complex cell interactions are noted in vitro where several different populations of cells alone or in collaboration can produce erythroid burst-promoting activities. The physiologic significance of these molecule-cell interactions remains to be determined.

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3A1 (CD7) Expression Precedes T_β Gene Rearrangements In Precursor T (Lymphoblastic) Neoplasms

By Stefania Pittaluga, Mark Raffeld, Edward H. Lipford, and Jeffrey Cossman

The phenotypes of early stages of T cell maturation are reflected by precursor T (lymphoblastic) neoplasms. In the present study, a series of such neoplasms was analyzed to reveal the developmental association of the expression of stage-related cell surface markers and T cell receptor gene rearrangement. Rearrangements of the T cell receptor β-chain (T_β) gene were found in most, but not all, cases (88%) of T cell lymphoblastic neoplasms. T_β gene rearrangement preceded surface expression of the T cell receptor-linked molecular complex T3. Of all monoclonal anti-T cell antibodies tested, only antibody 3A1 was capable of reacting with neoplastic cells from all cases irrespective of the occurrence of T cell receptor gene rearrangements. In contrast, markers T1 and T11, normally expressed by mature T cells, were absent from the neoplastic cells in many cases (73% and 60% positive cases, respectively). Thus, antibody 3A1 is a valuable probe for the identification of T lymphoblastic neoplasms since its target antigen is consistently expressed and does not require prior T_β gene rearrangement. Furthermore, expression of 3A1 prior to T_β gene rearrangement suggests that it may be a cell surface protein that participates in the triggering of T cell receptor gene rearrangement and expression. It is concluded that precursor T cell neoplasms display an early T cell development hierarchy that, in sequence, consists of 3A1 expression, T_β gene rearrangements, and surface T3 expression.

THE DIFFERENTIATION of T cell precursors into mature effector cells occurs in the thymus and is associated with the acquisition of functional cell surface membrane molecules. As a consequence, the developmental pathway of early T cells can be characterized by the detection of these sequentially expressed surface molecules using a series of monoclonal antibodies (e.g., T3, T6, T11, etc).[1] A critical feature acquired by T cells is their capacity to recognize unique antigens. This is achieved during thymocyte differentiation whereby genes encoding the specificity of the T cell antigen-specific receptor undergo rearrangement in a manner reminiscent of immunoglobulin genes in pre-B cells.[2-5] As thymic T cells mature, the translational products of the rearranged genes become assembled as an immunoglobulinlike heterodimer composed of one α and one β chain.[6] Expression of surface membrane T cell antigen receptors is thought to be dependent on the obligatory coexpression of and noncovalent linkage with the invariant protein T3.[7]

Neoplasms of immature T cells, lymphoblastic leukemias, and lymphomas express thymocyte phenotypes, have a striking propensity for intrathymic growth, and appear to be neoplastic counterparts of thymocytes arrested in distinct developmental stages.[1,8-12] Recognition of the precursor T cell phenotype of T lymphoblastic neoplasms has provided the basis for immunophenotypic analysis as a clinically critical diagnostic and classification tool.[11-14] Because of their immature T cell phenotype, we reasoned that lymphoblastic neoplasms might reflect phenotypes associated with the T cell receptor gene rearrangement process. Moreover, T cell neoplasms are clonal expansions, and consequently, their uniformly rearranged T cell receptor genes are readily amenable to restriction enzyme analysis.[15-20]

In the present study we have examined T cell receptor β-chain (T_β) gene rearrangements in a series of human precursor (lymphoblastic) T cell neoplasms to evaluate the developmental relationship between gene rearrangement and cell surface protein expression. Evidence presented demonstrates the diagnostic application of molecular genetics to precursor T cell neoplasms, since the majority have undergone T_β rearrangement and are clonal, T lineage cells. Moreover, the series of precursor T cell neoplasms displays an early T cell developmental hierarchy that in sequence, consists of 3A1 expression, T_β gene rearrangement, and T3 expression. Gene rearrangements in precursor T cell neoplasms underscore the valuable contribution of molecular genetics to diagnostic pathology and provide insight into the developmental events surrounding T_β gene rearrangement.

MATERIALS AND METHODS

Precursor T cell neoplasms. All lymphoproliferative processes were classified by conventional microscopy as either acute lymphoblastic leukemia or malignant lymphoma, lymphoblastic. Viable cell suspensions stored in the vapor phase over liquid nitrogen were used for cell surface marker studies and DNA extraction as described.[17] In each case >80% of cells were morphologically neoplastic, reacted with at least one anti-T, but not B1 and B4, monoclonal antibody, and contained terminal deoxynucleotidyl transferase as shown by antibody staining of fixed cytocentrifuge preparations, as previously outlined.[12,21]

Cell surface phenotyping. Washed, viable cells were treated with monoclonal antibodies and analyzed by indirect immunofluorescence in a fluorescence-activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, Calif) as described.[12,21] Cells were scored positive when >20% of the lymphoblasts reacted with antibody. Monoclonal antibodies and their antigen targets (in parentheses) were B1, B4, 3A1 (CD7), Leu-1 (T1, CD5), Leu-2 (T8, CD8), Leu-3 (T4, CD4), Leu-4 (T3, CD3), OKT6 (T6, CD1), OKT11 (T11, CD2), Lyt 3/9.6 (T11, CD2), J5 (common acute lymphoblastic leukemia antigen, CALLA, CD10), and HLA-DR. Antibodies were obtained from the following sources: B1, B4, and J5 (Coulter Immunology, Hialeah, Fla); OKT6 and OKT11 (Ortho Pharmaceutical Corp, Raritan, NJ); Leu series and HLA-DR (Becton Dickinson); Lyt 3/9.6 (New England Nuclear, Boston) and 3A1 (American Type Culture Collection, Rockville, Md).

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Southern blot hybridization. High–molecular weight genomic DNA was extracted from lymphoid cells and digested with the restriction endonucleases *Bam*HI, *Eco*RI, and *Hind*III in accordance with the directions of the manufacturer (New England Biolab, Beverly, Mass) as described previously.[21,22] Digested DNA was electrophoresed through 0.8% agarose gels and transferred to nylon filters (Gene-Screen Plus, New England Nuclear). Filters were hybridized overnight with ^{32}P-labeled nucleic acid probes containing 500 to 1,000 dpm/pg and washed at high stringency as outlined by the supplier. Autoradiograms were prepared by exposing filters to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) for one to seven days.

Nucleic acid probes. The C region was isolated from a T cell receptor β-chain cDNA YT35 (base pair [bp], 100 to 870, ref 2), by digestion with AvaI/PstI (C_β), and the 403-bp insert was subcloned into a pSP64 vector (Promega Biotec, Madison, Wis). Inserts were purified twice through low–melting point agarose and labeled with ^{32}P by random priming.

To detect Ig gene rearrangements, DNA probes of J_H, C_K, and C_λ were used as described.[21,22]

RESULTS

Characterization of the T_β gene in precursor T cell neoplasms. Restriction enzyme digestion with *Bam*HI and *Eco*RI and Southern blot analysis revealed frequent (14 of 16 cases) chain gene rearrangements in precursor T cell neoplasms (Table 1, Fig 1). In contrast, an Ig heavy-chain gene was rearranged in only one case, and Ig light chain genes were unrearranged in all T cell neoplasms. Only one of 30 B cell neoplasms contained T_β gene rearrangements (Fig 1). Rearrangements of the β-chain gene involved either a single allele (eight cases) or both alleles (six cases). Rearrangement of the second β-chain allele implies that T cell receptor gene rearrangements in these cells may be subject to error and require a second attempt at successful rearrangement, analogous to the mechanism used by B cells in the formation of functional Ig genes.[23]

A single restriction endonuclease, *Bam*HI, was sufficient to detect rearrangements in 13 of the 14 cases in which rearrangements occurred and, therefore, proved to be a useful enzyme for initial screening. It would be expected that *Bam*HI-digested DNA should show T_β gene rearrangements in most cases since the *Bam*HI restriction sites flank the entire $J_\beta1$-$C_\beta1$-$J_\beta2$-$C_\beta2$ complex, and the *Bam*HI restriction fragment would be altered by rearrangement involving either the $J_\beta1$ or $J_\beta2$ locus.[4] Additional rearrangements were detected with *Eco*RI in case 1, which showed no rearrangements with *Bam*HI, and in case 10, in which one rearrangement was detected with *Bam*HI and two with *Eco*RI. Presumably, rearranged bands obtained with *Bam*HI digestion comigrated with other bands on these blots. Demonstration of nongermline bands with *Eco*RI is indicative of a rearrangement of the $J_\beta1$-$C_\beta1$ complex, since the 12-kilobase (kb) *Eco*RI fragment encompassing this complex would be altered when $J_\beta1$ is involved in rearrangement. The 4-kb *Eco*RI germline fragment, which includes $C_\beta2$ but not $J_\beta2$, remains in the germline configuration regardless of the rearrangement of either the β1 or β2 complex.[24] No β-chain gene rearrangements were detected in two cases (2 and 3) despite digestion with *Bam*HI, *Eco*RI, or *Hind*III (Fig 2).

It should be noted that rearranged β-chain gene restriction fragment sizes differed from case to case. Therefore, it would appear that precursor T cell neoplasms may be using many different V_β genes and that no single common V gene is used as was previously suggested in a preliminary screening with *Eco*RI.[25]

Developmental association of phenotype and gene rearrangement. All cases, regardless of the occurrence of T cell receptor gene rearrangements, reacted intensely with the monoclonal antibody 3A1 and were unreactive with the B cell–associated antibodies B1 and B4. Thus, the 3A1+, B1−, B4− phenotype specifically distinguishes T and B cell precursor neoplasms since precursor B cell neoplasms are 3A1-negative[26] and react with antibodies B1 or B4.[27] Furthermore, the gene rearrangement studies presented here confirm that phenotypically defined precursor T cell neoplasms are uniformly 3A1+, B1−, and B4−.

Table 1. Phenotype and Genotype of Precursor T Cell Neoplasms

												C_β			J_H	
Case	B1	B4	3A1	T1	T3	T4	T6	T8	T11	CALLA	DR	B	E	H	B	E	H
1	−	−	+	+	−	−	+	+	+	+	−	G	GR			R	R
2	−	−	+	−	−	−	−	−	+	−	+	G	G	G		G	G
3	−	−	+	+	−	−	−	−	−	−	−	G	G	G	G	G	G
4	−	−	+	+	−	−	−	−	−	−	+		GR		G		
5	−	−	+	+	+	+	+	−	+	+	−	RR	R		G		
6	−	−	+	+	+	+	+	+	+	+	−	RR			G		
7	−	−	+									GR			G		
8	−	−	+	−	+	−	−	+	+	−	−	GR			G		
9	−	−	+	+	+	−	+/−	−	+	−	−	GRR			G		
10	−	−	+	+	+	+	+	−	+	−	−	GR	RR		G		
11	−	−	+	+	−	−	+	−	−	−	−	GR			G		
12	−	−	+	+	−	−	−	−	−	+	−	GRR			G		
13	−	−	+	−	−	−	−	−	−	−	+	GR	GR			G	G
14	−	−	+	+	+	−	−	−	−	+	+		R		G		
15	−		+	−	−	−	+	+	+	−	−	GRR			G		
16	−		+	+	+	+	−	+	+	−	−	GR			G		

−, <10%+; +/−, 10% to 50%; +, >50%+.

Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; G, germline; R, rearranged; RR, two alleles rearranged.

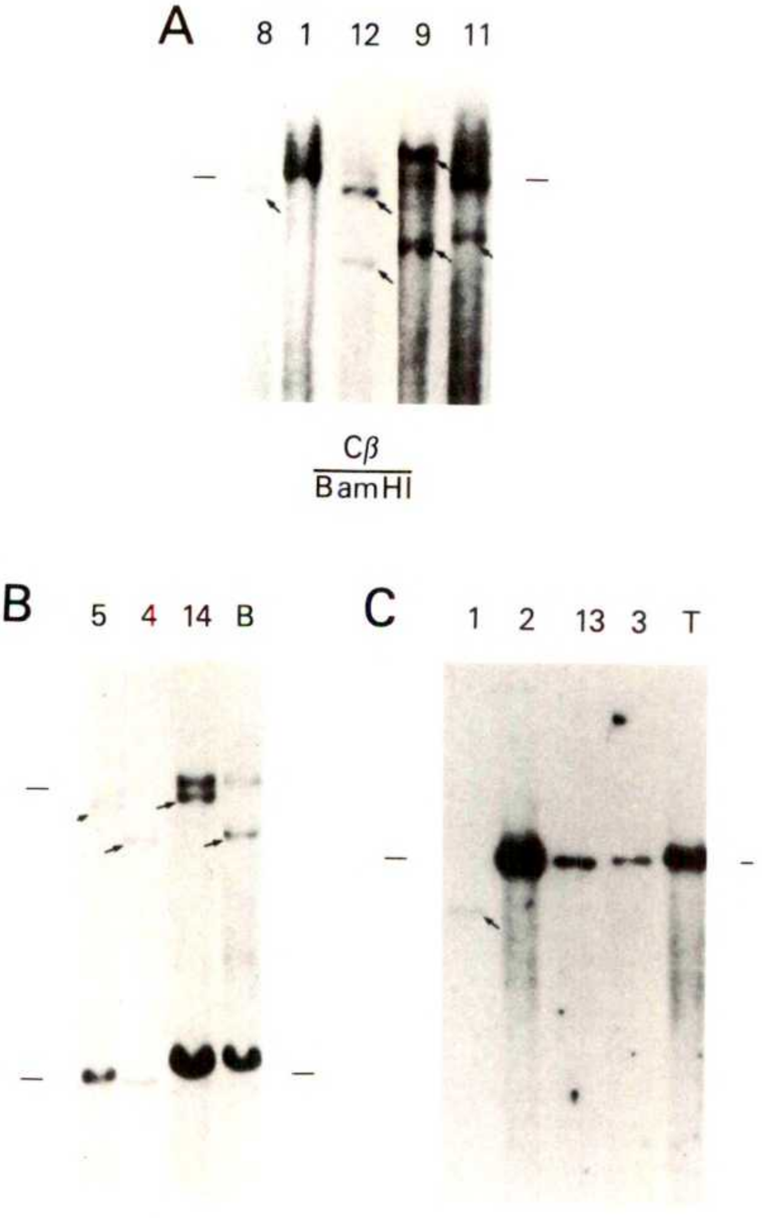

Fig 1. Gene rearrangements in precursor T cell neoplasms. Genomic DNA was digested with either *BamH*I (A) or *EcoR*I (B and C) and probed with the C_β cDNA (A and B) or a genomic probe of J_H (C). Lanes are labeled according to the case number presented in Table 1. Germline configuration of the genes is designated by a dash adjacent to the blot and rearrangements are shown by small arrows. In panel B the lane labeled B is a precursor B cell lymphoblastic leukemia, and in panel C for lane labeled T is normal human thymus as a control. A summary of the rearrangements is indicated in Table 1.

In contrast to the constant expression of the surface protein recognized by 3A1 in all cases, other molecules were selectively expressed. Surface T3 was expressed by seven of 15 cases tested and was preceded developmentally by T_β gene rearrangement, since rearrangements had occurred in all T3+ neoplasms and in six of eight T3− cases. It is known from studies of mutant T cell clones that the obligatory coexpression of T3 and the T cell receptor is contingent upon functional T cell receptor gene rearrangement and expression.[7] As a result, surface T3 is undetectable in the absence of T cell receptor gene rearrangement and expression. This fact, coupled with the requirement for T_β rearrangement prior to T3 expression presented here, would predict that T cell receptor surface expression in T cell neoplasms would adhere to the T3/T cell receptor coexpression rule.

A dissimilarity between precursor T cell neoplasms and normal cortical thymocytes is the expression of the sheep erythrocyte receptor or T11 molecule. This 50-kilodalton (kd) molecule, detected by monoclonal antibodies OKT11 and 9.6 (Lyt-3), is found on >95% of both cortical and medullary thymic lymphocytes.[1] However, six of 16 (37.5%) cases did not bear the T11 molecule, and included among these T11− cases are neoplasms with either rearranged or germline T_β genes.

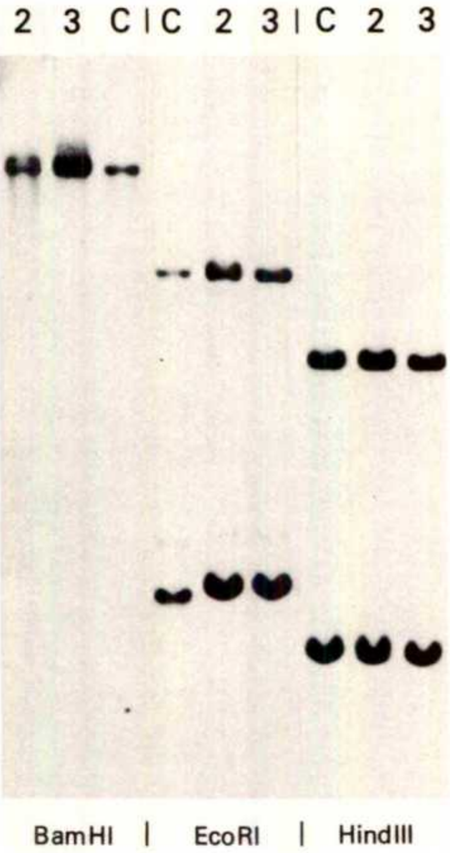

Fig 2. **Lack of T cell receptor β-chain gene rearrangements in two T lymphoblastic neoplasms. DNA from cases 2 and 3 and from a control (C, placenta) was digested with the indicated restriction enzymes and probed with the C_β cDNA fragment. No rearrangements of the β-chain gene were detected in either case 2 or case 3.**

DISCUSSION

By applying Southern blot analysis we found T_β gene rearrangements in most, but not all, precursor T cell lymphoblastic neoplasms. Rearrangements of the T_β gene were supportive of the T cell nature of these lymphoblastic neoplasms, since they only rarely occur in B cell neoplasms. In those instances where many conventional T cell–associated phenotypic markers were not expressed, T_β gene rearrangement analysis independently confirmed its T cell derivation. Another contribution of T_β gene analysis was the demonstration of monoclonality in T cell precursor neoplasms. Unlike B cells, T cells lack phenotypic markers for monoclonality, and therefore, the T cell receptor gene serves as a particularly useful parameter in the analysis of T cell lymphoproliferative disorders.[15–20]

Our gene rearrangement studies confirm that T_β gene rearrangements are not entirely restricted to T cells.[28,29] Lack of T_β rearrangements in most B cell neoplasms indicates that, despite close similarities in the organization of T_β and Ig gene recombination sequences, constraints governing rearrangement are generally regulated in a setting of T or B cell commitment. Out-of-context gene rearrangements of Ig heavy chain in malignant T lymphoblasts,[15,26,30,31] or T_β in B cell neoplasms, might represent isolated instances in which rearrangements occurred independently of lineage commitment and recombinatorial sequences from both genes were susceptible to the recombination mechanism. Rearrangements of both Ig heavy and light genes appear to occur specifically in B cells only.[21] It will be interesting to discover whether an analogous specificity is associated with T cell lineage, perhaps reflected by rearrangements of T_α and T_β genes.

T_β genes were retained in their germline, unrearranged organization in two of our T cell lymphoblastic neoplasms (Fig 2). Their T cell lineage was demonstrated by expression of 3A1 in both cases and T11 in case 2 and T1 in case 3. Neither showed any IgH gene rearrangements, and they did not express B1 or B4. Thus, these two cases appear to represent precursor T cells arrested at an immature stage of cortical thymocyte development prior to the onset of T_β gene rearrangement.

Expression of 3A1 by the two germline cases, as well as by all T lymphoblastic neoplasms having T_β gene rearrangements, underscores the importance of the use of 3A1 antibody for the identification of pre-T neoplasms.[13,32] 3A1 is not found on precursor B cell neoplasms[26] and, therefore, is a specific marker for the differential diagnosis of pre-T and pre-B neoplasia. 3A1 was the only marker expressed by all T cell lymphoblastic neoplasms in this series and in previous reports using either antibodies 3A1 or Leu-9, which apparently recognize the same surface molecule.[13] Significantly, these studies, as well as the present one, have shown that immature T cell neoplasms are often T11-negative. The frequency of T11– cases provides further support that a definition of precursor T cell neoplasms should not be restricted to T11+ cases but should encompass all 3A1+/Leu-9+ lymphoblastic leukemias and lymphomas.[13] The relatively common 3A1+, T11– precursor T cell neoplasm phenotype does not directly correlate with any of the known compartments of T cell differentiation, and when coexpression of other differentiation markers (T3, T4, T6, and T8) is taken into account, a rather broad range is represented by the 3A1+, T11– neoplasms (Table 1). Furthermore, T11 expression did not correlate with T_β gene rearrangement since both T11+ and T11– cases were found in either the presence or absence of rearrangements.

It has been suggested that the T11 molecule might be involved in triggering T_β gene rearrangement in T cell precursors,[33] but frequent absence of T11 in pre-T neoplasms implies that T11 is either transiently expressed or may be acquired after rearrangement has occurred. In this regard, the 40-kd surface polypeptide detected by antibody 3A1 might be considered a candidate surface molecule that plays a role in the development of rearrangements and their expression. However, 3A1/Leu-9 is not expressed by many cortical thymocytes or by 10% to 15% of mature T cells,[13,34] and its functional role awaits determination.

We have determined the pattern and occurrence of T cell receptor gene rearrangements in pre-T neoplasms and related these genetic findings to cellular phenotype. Precursor T cell neoplasms were found to encompass the earliest stages of T cell differentiation associated with the development of T_β gene rearrangements. Immunophenotypic and genetic characterization of this class of lymphoid neoplasia not only provides clues concerning their cellular differentiation but should also open new avenues for precise diagnosis and classification of T lymphoproliferative disease.

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The Effect of Platelets in the Activation of Human Blood Coagulation Factor IX by Factor XIa

By Hans Soons, Truus Janssen-Claessen, H. Coenraad Hemker, and Guido Tans

We report here the effect of activated human platelets on the activation of human factor IX by human factor XIa. Factor IXa formed during activation was determined via its ability to activate bovine factor X. To increase sensitivity, phospholipids and bovine factor VIIIa were present in the assay. The kinetic parameters of the factor IX activation were determined in the presence of 10 mmol/L $CaCl_2$. The K_m for factor IX was 0.30 μmol/L and k_{cat} was 2.4 s^{-1}. Activated human platelets inhibited factor IX activation by factor XIa in a dose-dependent manner, whereas unstimulated platelets had no effect. Factor IX activation was inhibited for more than 90% at a platelet concentration of 4 × 10^8/mL, whereas concentrations of less than 10^8/mL had no influence. The inhibitory effect could be induced by thrombin, collagen, calcium ionophore A 23187, and adrenalin. The appearance of inhibitory activity could be blocked by the addition of the prostacyclin analogue ZK 36374 at any time during platelet activation. Stirring during platelet activation was not necessary. These results suggest that the inhibition is caused by a release reaction. This was confirmed by centrifugation experiments that showed that the inhibitory activity could be recovered from the supernatant of the activated platelets. The inhibitory activity was destroyed upon boiling and was susceptible to trypsin digestion. Passage of platelet supernatant over ACA 22 showed that the inhibitory activity eluted with an apparent molecular weight of less than 1,200,000 but greater than 669,000. The inhibition of factor XIa was reversible. These data suggest that platelets release an antiprotease of factor XIa that reversibly inhibits factor XIa. Lineweaver-Burk analysis showed that the inhibitor caused both an increase in K_m for factor IX and a decrease in k_{cat} of factor IXa formation by factor XIa.

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HUMAN FACTOR IX is a vitamin K-dependent plasma protein that participates in the intrinsic pathway of blood coagulation.[1] It is present in plasma as a zymogen with a molecular weight (mol wt) of 57,000 daltons (d) at a concentration of 2.6 to 5.0 μg/mL. Human factor IX is converted to a serine protease, factor IXa, through proteolysis of two internal peptide bonds. This results in the release of an activation peptide (11,000 d) from the internal region of the precursor molecule. The resulting factor IXa (46,000 d) consists of a light chain (18,000 d) and heavy chain (28,000 d) held together by a disulfide bond(s).[2-3] Activation of factor IX in plasma can be accomplished by factor XIa[2-3] or by the factor VIIa-tissue thromboplastin complex.[4-5]

Platelets play an important role in blood coagulation. The platelet surface strongly promotes prothrombin and intrinsic factor X activation.[6] The activation of factor IX by factor XIa is one of the few reactions of coagulation for which no surface requirement is known. It has been suggested that collagen-stimulated platelets exhibit factor XIa-like activity, which can be determined in a clotting test.[7-8] However, others have failed to confirm this,[9] and it is not known whether factor XIa is involved. Up till now nothing is known about whether platelets have any effect in the activation of factor IX by factor XIa.

Several assays have been described in literature for the determination of factor IX activation: a coagulation assay in factor IX-deficient plasma,[10-12] a tritiated activation peptide release assay,[5,10,13] an immunoradiometric assay,[11] and an assay in which factor IXa is measured indirectly by determining the initial rate of factor X activation.[14] The most widespread used is the tritiated activation peptide release assay,[5,10,13] which was originally described for the activation of factor X by Silverberg et al.[15] In highly purified systems the appearance of factor IXa procoagulant activity follows the peptide release by a small lag period.[10] However, in less-purified systems, other enzymes may be present that give rise to similar peptide fragments but destroy the procoagulant activity of factor IX.[16] Therefore, to circumvent these potential difficulties, we have chosen to use an assay for factor IX activation by factor XIa, which detects the functional factor IXa activity via its ability to activate bovine factor X in the presence of phospholipids and $CaCl_2$.[14] To increase its sensitivity, bovine factor VIIIa was present. In that case, however, care has to be taken to add factor VIIIa under rigorously standardized conditions, since the assay is critically dependent on the amounts of factor VIIIa present.

This study was undertaken to explore the role of platelets in factor IX activation by factor XIa. We present evidence that platelets, upon stimulation, secrete an inhibitor that interferes with factor IX activation by factor XIa.

MATERIALS AND METHODS

Materials. Chromogenic substrates L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide hydrochloride (S2337) and *N*-benzoyl-L-isoleucyl-L-glutamyl-(piperidyl)-glycyl-L-arginine-*p*-nitroanilide hydrochloride S2366 were purchased from A.B. Kabi Diagnostica, Stockholm. The prostacylin analogue ZK 36374 was a kind gift from Schering AG, Berlin. Plasma deficient in coagulation factors IX, XI, or XII were from patients congenitally deficient in these factors and were obtained from George King Biomedical, Overland Park, Kan. Hormon tendon collagen was obtained from Hormon-Chemi, Munich. Fatty acid-free human serum albumin and trypsin were from Sigma Chemical Co, St Louis. Two-milliliter plastic flat-bottom reaction tubes were obtained from Sterilin Ltd, Teddington, England; Teflon-coated magnetic stirring bars (7 × 2 mm) were purchased from Bel-Art Products, Pequannock, NJ. All reagents used were of the highest grade commercially available.

Proteins. Human factor IX was purified based on a modification of the isolation procedure described by di Scipio et al.[17] and the sulfated dextran column as described by Miletich et al[18] was added. Citrated fresh-frozen human plasma was thawed, and benzamidine

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(10 mmol/L), heparin (6 mg/μL), and soybean trypsin inhibitor (10 mg/L) were added. The barium citrate absorption was followed by ammonium sulfate precipitations (40% and 70% saturation) before the vitamin K–dependent protein preparation was applied to a diethyl aminoethyl (DEAE)-Sephadex column followed by a heparin-Sephadex column.[17] Finally, a sulfated dextran column was used to purify factor IX.[18] Human factor IXa was prepared from purified human factor IX as described earlier for bovine factor IXa.[19] Human factor XI was purified according to Bouma et al.[20] Human factor XII was isolated as described by Griffin and Cochrane.[21] β-Factor XIIa was prepared from the purified human factor XII as described by Fujikawa and McMullen.[22] Bovine factor X was purified according to Fujikawa et al.[23] Bovine factor VIIIc was prepared as described earlier.[24] Factor VIIIa was prepared by activation of factor VIIIc (1.75 nmol/L) with thrombin (4 nmol/L) for one minute at 37 °C.[19,24] Bovine thrombin was purified as a prothrombin activation product as described by Rosing et al.[25] The specific activity of the thrombin preparation was 1 National Institutes of Health (NIH) U/mL = 11 nmol/L when a molecular weight of 35,600 was used for thrombin. The human factor IX, XI, and XII and the bovine factor X_1 and X_2 preparations were homogeneous and pure as determined by gel electrophoresis in the presence of sodium dodecyl sulfate on 10% gels according to Laemmli.[26] The specific activities of the protein preparation as determined with a clotting assay were 193, 211, 74, and 100 U/mg for human factors IX, XI, XII, and bovine factor X, respectively, assuming one unit of human factor IX, XI, and XII to be present per milliliter of human normal plasma and one unit of bovine factor X in one milliliter of bovine normal plasma. All proteins were stored at −70 °C after dialyzing against the following buffers: 50 mmol/L Tris-HCl, 175 mmol/L NaCl at pH 7.9 for human factor IX and bovine factor X; 4 mmol/L sodium citrate, 2 mmol/L acetic acid, 0.15 mol/L NaCl, and 0.5 mmol/L EDTA at pH 5.0 for human factor XI and XII; and 10 mmol/L morpholino ethane sulfonic acid, 175 mmol/L NaCl, 10 mmol/L $CaCl_2$, and 10% glycerol at pH 6.5 for bovine factor VIII. Trypsin was insolubilized by coupling trypsin (60 mg) in 0.1 mol/L $NaHCO_3$ to CNBr-activated Sepharose 4B (3 g) at 4 °C overnight according to the manufacturer's instructions.

Preparation of human factor XIa. Human factor XIa was prepared from human factor XI using human β-factor XIIa.[27] To 10 mL of factor XI (0.15 mg/mL) in 4 mmol/L sodium acetate, 2 mmol/L acetic acid, 0.15 mol/L NaCl, pH 5.0, was added 1.25 mL 0.5 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 8.0, at 37 °C. Reaction was started by adding 1.27 mL β-factor XIIa (0.59 mg/mL) in 4 mmol/L sodium acetate, 2 mmol/L acetic acid, 0.15 mol/L NaCl at pH 5.0. Factor XI activation was followed by measuring factor XIa amidolytic activity. Activation was completed within 90 minutes. Factor XIa was separated from β-factor XIIa on a DEAE-Sephadex column (1.5 × 11.5 cm) at 4 °C in 50 mmol/L Tris-HCl and 150 mmol/L NaCl at pH 8.0. Factor XIa did not adhere to the resin, whereas β-factor XIIa was eluted by a linear salt gradient (0.1 to 0.5 mol/L) NaCl.

The amidolytic activity of human factor XIa was measured using the chromogenic substrate S2366 in a buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 20 mmol/L EDTA, and 0.5 mg/mL human serum albumin, pH 7.9. The kinetic parameters of S2366 hydrolysis by human factor XIa were K_m = 0.42 mmol/L and k_{cat} = 758 s^{-1}. k_{cat} was calculated using a concentration of factor XIa based on a mol wt of 160,000.

Protein concentrations. Determinations of protein concentrations were routinely done according to Bradford.[28] Factors IXa, Xa and thrombin were determined by active-site titration with p-NPGB-p-nitrophenyl-p′-guanidinobenzoate (p-NPGB) as described earlier.[14,25] Concentrations of factor IX and factor X were determined by complete activation and subsequent determination of the active enzyme. Factor VIIIa concentrations were determined according to the method of van Dieijen et al.[24] Factor XIa concentrations were determined from the amount of protein present assuming a mol wt of 160,000 d.[25]

Phospholipid vesicles. Phospholipid vesicles were made from a mixture of 40 mol/100 mL 1,2-dioleoyl-sn-glycero-3-phosphoserine (PS) and 60 mol/100 mL 1,2-dioleolyl-sn-glycero-3-phosphocholine (PC) in a buffer containing 50 mmol/L Tris-HCl and 175 mmol/L NaCl at pH 7.9 as described by Rosing et al.[25]

Collagen. Collagen was added in the nonfibrillar form, dissolved in acidic dilution buffer obtained from Hormon-Chemie as described by Rosing et al.[6] The minimal amount of collagen inducing aggregation and secretion was 1 to 2 μg/mL.

Factor IXa determinations. To follow the activation of factor IX by factor XIa an assay system was developed in which factor IXa was determined via its ability to activate bovine factor X in the presence of $CaCl_2$, phospholipid, and factor VIIIa. Factor IXa–containing samples were diluted in ice-cold buffer containing 50 mmol/L Tris (pH 7.9), 175 mmol/L NaCl, 0.5 mg/mL human serum albumin, and 20 mmol/L EDTA to block further activation of factor IX. The amounts of factor IXa present in the samples were then determined as follows. Three hundred five microliters buffer containing 50 mmol/L Tris (pH 7.9), 175 mmol/L NaCl, 9.8 mmol/L $CaCl_2$, 0.5 mg/mL human serum albumin, and 16.4 μmol/L phospholipid vesicles was prewarmed at 37 °C for 4½ minutes, after which 45 μL bovine factor X (16.67 μmol/L) and a 50-μL sample were added. Factor X activation was started by adding 100 μL factor VIIIa (1.75 nmol/L). The final reaction mixture (500 μL) contained 50 mmol/L Tris (pH 7.9), 175 mmol/L NaCl, 6 mmol/L $CaCl_2$, 2 mmol/L EDTA, 10 μmol/L phospholipid, 1.5 μmol/L factor X, 0.35 nmol/L factor VIIIa, 0.5 mg/mL human serum albumin, and the factor IXa present in the sample. The rate of factor X activation was determined by measurement of the amounts of factor Xa formed after one and two minutes in these reaction mixtures using the factor Xa–specific chromogenic substrate S2337 as described earlier.[14,19] From the observed rate of factor X activation, the amount of factor IXa present in the sample was calculated using a calibration curve made with known amounts of active-site titrated factor IXa (see also Fig 1).

Kinetic parameters. K_m for factor IX and k_{cat} of factor IX activation were determined by measurement of the rate of factor IXa formation in a reaction mixture containing 10 mmol/L $CaCl_2$, 0.5 mg/mL human serum albumin, 0.1 nmol/L factor XIa, and varying amounts of factor IX in HEPES buffer, pH 7.5, using the assay previously described. K_m and V_{max} (expressed as factor IXa formed per second) were then determined by statistical analysis of the data as described by Eisenthal and Cornish-Bowden.[29] k_{cat} was calculated by dividing V_{max} with the enzyme concentration assuming a mol wt of 160,000 for factor XIa.

Isolation of human platelets. Blood was drawn by venipuncture from healthy male volunteers who had not taken any medication for at least ten days. As anticoagulants, 1 vol of acid citrate dextrose (0.18 mol/L glucose, 0.08 mol/L trisodium citrate, and 0.052 mol/L citric acid) was added to 5 vol of whole blood. Platelet-rich plasma was obtained after centrifugation at 220 g for 25 minutes. Platelets were isolated by centrifugation at 1000 g for 20 minutes, and the pellet was gently resuspended in calcium-free HEPES buffer (10 mmol/L HEPES, 136 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L $MgCl_2$, and 5 mmol/L glucose), pH 6.7, containing 0.4% human serum albumin. The platelets were washed twice in this buffer by centrifugation at 600 g for 15 minutes. During the whole procedure except the last wash, acid/citrate/dextrose was present in the buffer (1 vol to 14 vol buffer).[30] Finally, the platelets were resuspended in HEPES buffer, pH 7.5, containing 0.1% human serum albumin. Platelet concentrations were determined with a Coulter counter

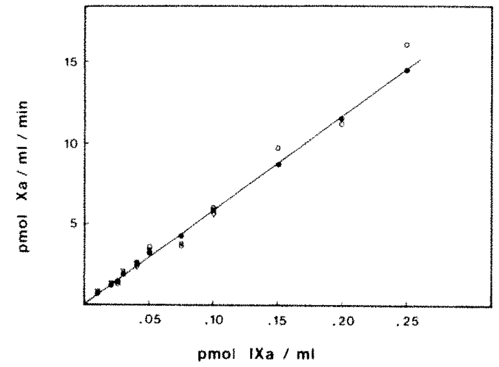

Fig 1. Factor IXa calibration curve. The rate of activation of bovine factor X (1.5 μmol/L) by varying amounts of factor IXa was determined in the absence (●) or in the presence of 3.2 × 10^7/mL stimulated platelets (▽) or their supernatants (○) at 37 °C in a reaction volume of 500 μL containing 50 mmol/L Tris (pH 7.9), 175 mmol/L NaCl, 6 mmol/L $CaCl_2$, 2 mmol/L EDTA, 10 μmol/L phospholipid vesicles (40% PS/60% PC, mole/mole), 1.5 μmol/L factor X, 0.35 nmol/L factor VIIIa, 0.5 mg/mL human serum albumin, and amounts of factor IXa as indicated in the figure. When stimulated platelets or their supernatants were present, factor IXa was preincubated with the platelets or their supernatant for four minutes at 37 °C prior to addition to the assay. Stimulated platelets were prepared by incubation at 37 °C for 12 minutes with 1.2 nmol/L thrombin, 10 μg/mL collagen, and 3 mmol/L $CaCl_2$ under continuous stirring. The supernatants were prepared by centrifuging the stimulated platelets for five minutes in an Eppendorf microfuge. For experimental details, see Materials and Methods under Factor IXa determination.

(Coulter Electronics, Luton, England) and were kept at room temperature.

RESULTS

Validation of the assay for factor IXa. In this study an assay for the activation of factor IX was used in which functional factor IXa activity was determined via the activation of bovine factor X as described earlier.[14] To increase its sensitivity, the activation of bovine factor X by human factor IXa was performed in the presence of $CaCl_2$, phospholipids, and bovine factor VIIIa. The amount of factor Xa formed was linear with time for at least two minutes (data not shown), and the rates of factor Xa formation were determined from the amounts of Factor Xa formed during these two minutes. Figure 1 shows that the rate of factor Xa formation was proportional to the amount of factor IXa added (closed circles). The slope of this plot reflects the rate of factor Xa formation per factor IXa present (59.5 nmol/Xa/min/nmol IXa). An excellent day-to-day reproducibility of the calibration curve could be achieved, since four separate experiments carried out at different days showed an SD of 1.7% in the slope of this plot.

This calibration curve was used in the experiments in which the activation of factor IX by factor XIa was determined. Since the experiments presented here concern the effect of platelets on the activation of factor IX by Factor XIa, the calibration curve was repeated in the presence of 3.2 × 10^7/mL thrombin/collagen-stimulated platelets (Fig 1, open triangles) or the supernatant thereof (Fig 1, open circles). As can be seen, the presence of the triggered platelets or their supernatants had no effect on the assay for factor IXa.

Factor IX activation by factor XIa. The assay just described was used to assess the kinetic parameters of factor IX activation by factor XIa in the presence of 10 mmol/L $CaCl_2$. It was verified that factor IX activation was linear with time and proportional to the amount of factor XIa used (data not shown). Varying concentrations of factor IX were incubated with factor XIa, and the rate of factor IXa formation was determined in the assay just described. Michaelis-Menten kinetics were observed throughout. The kinetic parameters were K_m = 301 nmol/L, and k_{cat} = 2.4 s^{-1} assuming a mol wt of 160,000 for factor XIa.

The effect of platelets in the activation of factor IX by factor XIa was studied. Nonstimulated platelets up to concentrations as high as 5 × 10^8/mL had no effect on factor IX activation. However, in the presence of platelets stimulated by the combined action of thrombin and collagen, the rates of factor IXa formation decreased with time until after two minutes a low rate of factor IXa formation remained (Fig 2). Preincubation of factor IX with the activated platelets for four minutes did not change the time course of factor IX activation. However, when factor XIa was preincubated with

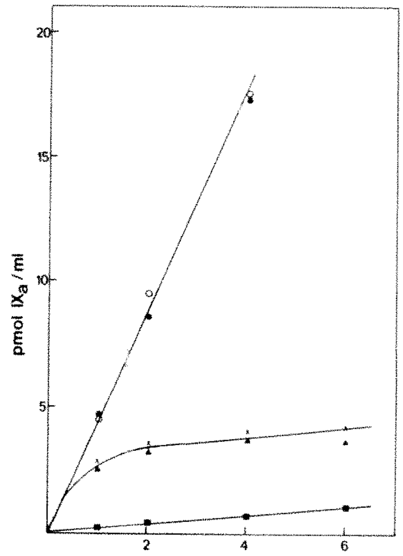

Fig 2. The effect of platelets in the activation of factor IX by factor XIa. The activation of factor IX (100 nmol/L) by factor XIa (0.1 nmol/L) was determined at 37 °C in the absence of platelets (○), in the presence of 5 × 10^8/mL unstimulated platelets (●), and in the presence of platelets (5 × 10^8/mL) that had been stimulated for 12 minutes at 37 °C under continuous stirring by thrombin (1.2 nmol/L), collagen (10 μg/mL), and 3 mmol/L $CaCl_2$ (X). The final reaction mixture contained 10 mmol/L $CaCl_2$, 0.5 mg/mL human serum albumin, 100 nmol/L factor IX, 0.1 nmol/L factor XIa, and 5 × 10^8/mL platelets in HEPES buffer at pH 7.5. At the time intervals indicated, samples were withdrawn and assayed for factor IXa as described in Materials and Methods. The points denoted (▲) were obtained after factor IX was incubated four minutes with the stimulated platelets prior to the addition of factor XIa. The points denoted by (■) were obtained after a four-minute incubation of factor XIa with the stimulated platelets prior to the addition of factor IX.

the triggered platelets, only the low rate of factor IXa formation remained. This strongly suggests that the inhibition was the result of a direct interaction with factor XIa that was completed within four minutes. Subsequent experiments showed that for platelet concentrations ranging from 10^6 to 10^9/mL a preincubation time with factor XIa of approximately ten minutes sufficed to result in linear rates of factor IXa formation. Therefore, in all subsequent experiments, factor XIa was preincubated for ten minutes with the platelets prior to the addition of factor IX.

The inhibitory effect of activated platelets at a suboptimal platelet concentration (4.10^7/mL) in factor IX activation by factor XIa was studied using different platelet agonists (Table 1). The most potent were thrombin (1.2 nmol/L), calcium ionophore A 23187 (1 μmol/L) and the combined action of thrombin (1.2 nmol/L) and collagen (10 μg/mL), whereas platelets activated by collagen (10 μg/mL) or adrenalin (546 μmol/L) caused a smaller inhibition. Platelets stimulated with adenosine diphosphate (ADP) (25 μmol/L) whether in the absence or presence of fibrinogen (0.7 mg/mL), showed no significant inhibition of factor IX activation by factor XIa under these conditions. Stirring of the platelets was not necessary to achieve the inhibitory activity.

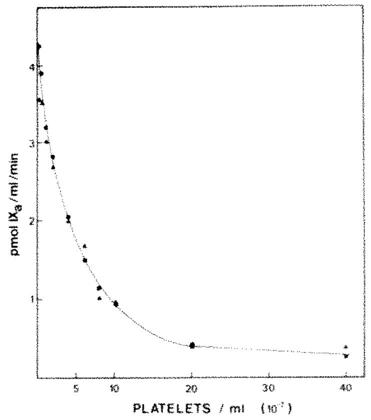

Fig 3. **Factor IX activation in the presence of varying concentrations of activated platelets or their supernatants. Varying amounts of platelets were stimulated for 12 minutes at 37 °C under continuous stirring with thrombin (1.2 nmol/L) and collagen 10 μg/mL) in the presence of 3 mmol/L $CaCl_2$. The platelets were then either incubated for an additional five minutes or centrifuged for five minutes in an Eppendorf Microfuge to obtain the supernatant. After this, the $CaCl_2$ concentration was adjusted to 10 mmol/L, and factor XIa was added and incubated for an additional ten minutes with the platelet suspensions or the supernatants. Subsequently, factor IX was added, and the rate of factor IXa formation was determined as described in Materials and Methods. The final reaction mixtures contained 10 mmol/L $CaCl_2$, 0.5 mg/mL human serum albumin, 100 nmol/L factor IX, and 0.1 nmol/L factor XIa in HEPES buffer (pH 7.5) and amounts of platelets (●) or their supernatants (▲) as indicated in the figure.**

Figure 3 shows that factor IX activation was inhibited in a dose-dependent way. Factor IX formation was not influenced at platelet concentrations less than 10^6/mL, and at higher platelet concentrations inhibition was observed until at a concentration of 4×10^8/mL factor IX activation was almost completely inhibited. During the platelet activation the lysis was always smaller than 2%. Lactic dehydrogenase (LDH) was taken as the parameter for platelet lysis. One hundred percent lysis was the amount of LDH measured when the platelets were sonicated. When the triggered platelet suspension was centrifuged, all the inhibitory activity was recovered in the supernatant (Fig 3), indicating that the inhibition is the result of platelet secretion upon triggering.

Table 1. The Effect of Different Platelet Stimulators in Factor IX Activation by Factor XIa

Trigger Substance	Rate of Factor IX Activation
No trigger	100%
ADP	95%
ADP/fibrinogen	92%
Adrenalin	84%
Collagen	84%
Thrombin	62%
A23187	58%
Thrombin/collagen	59%
Thrombin/collagen*	58%

*The platelets were activated by thrombin (1.2 nmol/L) and collagen (10 μg/mL) without stirring.

Platelets (4×10^7/mL) were incubated in HEPES buffer (pH 7.5) at 37 °C for 12 minutes in the presence of ADP (25 μmol/L), ADP (25 μmol/L), human fibrinogen (0.7 mg/mL), adrenalin (546 μmol/L), collagen (10 μg/mL), ionophore A23187 (1 μmol/L), thrombin (1.2 nmol/L), or thrombin (1.2 nmol/L), and collagen (10 μg/mL). During stimulation the platelets were stirred. After this incubation, factor XIa was added and incubated for ten minutes with the activated platelet suspension. The factor IX activation mixture contained 4×10^7/mL platelets, 100 nmol/L factor IX, 0.1 nmol/L factor XIa, and 10 mmol/L $CaCl_2$ in HEPES buffer, pH 7.5. Factor IX activation rates were determined as described in Materials and Methods and expressed as a percentage of the activation rate measured in the presence of unstimulated platelets (3.58 pmol IXa/min/mL).

The release of inhibition was studied using the prostacyclin analogue ZK 36374. This substance effectively blocks any further platelet aggregation[31] and platelet release reactions[32] when added to platelet suspensions (Fig 4). The addition of 100 nmol/L prostacyclin analogue prior to stimulation with 0.2 nmol/L thrombin effectively blocked any release of inhibitory activity. The time course of release of inhibitor was determined by the addition of ZK 36374 at various time intervals after the addition of thrombin to the platelets. The amounts of inhibitory activity released were then determined in the supernatant after centrifugation. Figure 5 shows that secretion of the inhibitor started after a small lag period of 10 seconds and reached a plateau after a platelet activation time of approximately four minutes.

Preliminary characterization of the inhibitory activity. To gain insight into the nature of the inhibitory activity secreted by platelets, the supernatant of a large amount of stimulated platelets (1.8×10^{10}) was collected, concentrated, and passed over a Sepharose ACA 22 column (2.5 × 85 cm). Figure 6 shows that the inhibitory activity was included in the column volume but eluted earlier than the largest mol wt marker available (669,000), thus precluding an accurate estimation of the apparent mol wt. The fractions of the column containing inhibitor were pooled and

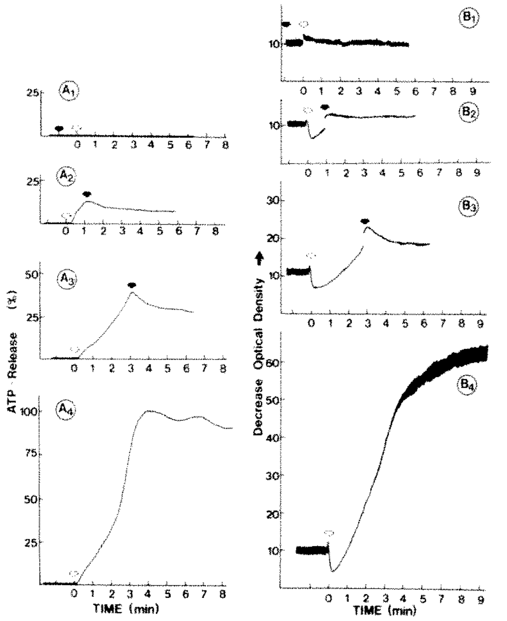

Fig 4. Influence of ZK 36374 on platelet aggregation and adenosine triphosphate (ATP) release. Platelet aggregation and ATP release were measured in an aggregometer. Washed platelets (2.3×10^8/mL) in HEPES buffer containing 2.6 mmol/L $CaCl_2$ and luciferin luciferase reagents were placed in the aggregometer under stirring conditions. At time 0, platelet activation was started by adding (0.7 nmol/L, ie 0.06 U/mL) thrombin (open arrow). At different time points, ZK 36374 (90 nmol/L) was added to stop the platelet activation (closed arrow). ATP release was monitored by luciferin-luciferase reaction (A), and platelet aggregation was measured at the same time (B). When ZK 36374 was added one minute before thrombin, no ATP release and platelet aggregation was measured (A_1 and B_1). When ZK 36374 was added one or three minutes after thrombin, ATP release and aggregation stopped immediately (A_2, B_2, and A_3, B_3). ATP release and platelet aggregation were also measured in the absence of ZK 36374 (A_4 and B_4). ATP release is expressed as a percentage, with ATP release in the absence of ZK 36374 taken as 100%.

concentrated, after which a part was treated with trypsin-Sepharose. Table 2 shows that no inhibitory activity remained after this treatment, whereas a control experiment with only Sepharose showed no loss of inhibitory activity. When the experiment with the trypsin-treated inhibitor was repeated in the absence of factor XIa, no activation of factor IX was observed, indicating that no significant amounts of trypsin were coming off the beads (data not shown). The inhibitor was also destroyed when placed in a boiling water bath for one hour. From these data we conclude that the inhibitor is a protein or contains a protein part essential for its activity that is susceptible to trypsin and is heat labile.

The data in Table 3 show that the inhibitor did not destroy or irreversibly inhibit factor XIa. When factor XIa was incubated with a high amount of inhibitor, only 21% of the rate of factor IX activation remained. However, when this incubation mixture was diluted fivefold, the rate of factor IX activation was only 1.6-fold less. Since rates of factor IX activation were linear with the amount of factor XIa present (see also the aforementioned), this indicated that some 50% of the factor XIa had become available again to participate

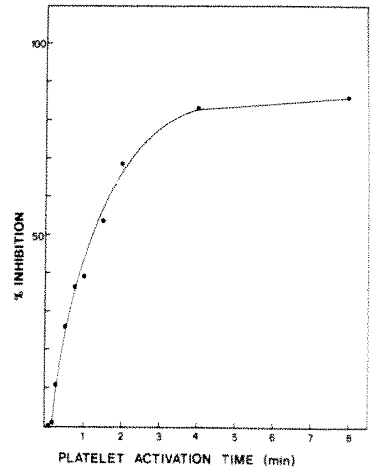

Fig 5. Time course of the appearance of inhibitory activity during platelet activation. Platelets (2.6×10^8/mL) were stimulated at 37 °C with thrombin (0.2 nmol/L) in the presence of $CaCl_2$ (3 mmol/L) under continuous stirring. At the time points indicated, the release reaction was blocked by adding the prostacyclin analogue ZK 36374 (100 nmol/L), and the platelet suspension was centrifuged for two minutes in an Eppendorf Microfuge to obtain the supernatant. After this, the $CaCl_2$ concentration was adjusted to 10 mmol/L, and factor XIa was added and incubated with the supernatant for 20 minutes at 37 °C, after which factor IX activation was started with the addition of factor IX. The final activation mixture contained 10 mmol/L $CaCl_2$, 0.5 mg/mL human serum albumin, 100 nmol/L factor IX, 0.1 nmol/L factor XIa, and supernatant of activated platelets (2×10^8/mL) in HEPES buffer. The rate of factor IX activation in the reaction mixture was determined as described in Materials and Methods and was expressed as a percentage of the factor IX activation rate measured in the presence of unstimulated platelets.

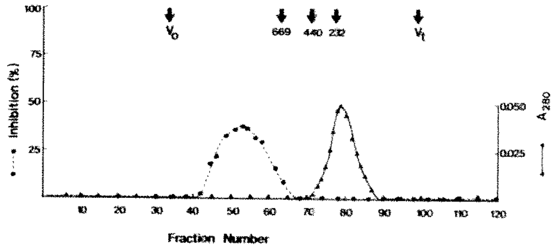

Fig 6. ACA 22 gel filtration on the supernatant of stimulated platelets. Platelets (1.15×10^9/mL) were activated in the presence of 3 mmol/L $CaCl_2$ with 1.2 nmol/L thrombin and 10 µg/mL collagen at 37 °C for 15 minutes in a total volume of 32.5 mL HEPES buffer (pH 7.5) containing 0.175 mg/mL human serum albumin. The supernatant was obtained by centrifugation for 20 minutes at 15,000 rpm in a Beckman J21 B and subsequently concentrated eightfold on a PM 10 filter (Amicon, Lexington, Mass). Two milliliters were applied to an ACA 22 column (2.5×85 cm) equilibrated in HEPES buffer (pH 7.5) at 4 °C. Elution was performed with the same buffer at a flow rate of 40 mL/h, and fractions of 4.5 mL were collected. Inhibitory activity was determined by incubating 450 µL of each fraction with 25 µL factor XIa (4nmol/L) for 15 minutes at 37 °C, after which 25 µL S2366 (5.8 mmol/L) was added and the rate of absorbance change at 405 nm recorded. The percentage of inhibitory activity was calculated from the amidolytic activity determined in the absence of inhibitor taken as 100%. Calibration of the column was achieved in a separate run using dextran blue 2000 (V_0), thyroglobulin (669,000), ferritin (440,000), catalase (232,000), and benzamidine (V_t).

Table 2. The Effect of Trypsin on the Activity of the Inhibitor in Factor IX Activation by Factor XIa

Activator	Rate of Factor IX Activation (pmol IXa/min/mL)
Factor XIa	3.97
Factor XIa, inhibitor	0.61
Factor XIa, inhibitor after incubation with Sepharose 4B	0.63
Factor XIa, inhibitor after incubation with trypsin-Sepharose	4.06

Human factor IX was activated by human factor XIa at 37 °C in HEPES buffer (pH 7.5) containing 100 nmol/L factor IX, 0.1 nmol/L factor XIa, 0.5 mg/mL human serum albumin, and 10 mmol/L $CaCl_2$ in the absence or presence of inhibitor. The rate of factor IXa formation was determined as described in Materials and Methods. Factor XIa was preincubated with inhibitor for 15 minutes at 37 °C before addition to the reaction mixture. The effect of trypsin on the inhibitor was determined by incubating 1.0 mL inhibitor with 0.25 mL trypsin-Sepharose slurry (or Sepharose 4B as a control) at 4 °C. After two hours, the suspension was centrifuged and the supernatant added to factor XIa to determine the residual inhibitory activity. The inhibitor preparation used was obtained from the ACA-22 column shown in Fig 6. Fractions 48 to 58 of this column contained inhibitory activity and were pooled. Human serum albumin, 2.5 mg, was added to the pool (44 mL), which was subsequently concentrated tenfold before use in the experiment described here.

in factor IX activation. The experiment in Fig 7 also illustrates this point. Here the time course of the disappearance of factor XIa amidolytic activity towards the chromogenic substrate S2366 was followed in an incubation mixture containing 0.4 nmol/L factor XIa and the supernatant of 2.2 × 10^8/mL activated platelets (closed squares). After equilibrium was reached, part of this mixture was diluted fivefold, and the remaining activity (expressed as a percentage of the amount of factor XIa present) was followed with time (closed circles). As can be seen, the amidolytic activity increased in the diluted mixture until the same final level was found as in a mixture that had been diluted fivefold at time 0 (closed triangles). Factor XIa activity was stable throughout the time course of the experiment in both the diluted and undiluted mixtures in the absence of platelet supernatant (open symbols). These data indicate that the inhibition of factor XIa was completely reversible.

Table 3. Effect of Dilution on the Activity of the Inhibitor in Factor IX Activation by Factor XIa

Activator	Rate of Factor IXa Formation (pmol IXa/min/mL)	
	Before Dilution	After Dilution
Factor XIa (0.1 nmol/L)	3.77	0.69
Factor XIa (0.1 nmol/L), supernatant of 2 × 10^8/mL platelets	0.80	0.49

Factor XIa (0.11 nmol/L) was incubated at 37 °C in HEPES buffer (pH 7.5) containing 1 mg/mL human serum albumin and 10 mmol/L $CaCl_2$ in the presence or absence of supernatant from 2.2 × 10^8/mL stimulated platelets (obtained as described in the legend to Fig 6). After 15 minutes, part of this mixture was diluted fivefold in the same buffer and further incubated at 37 °C for 30 minutes to allow reequilibration of factor XIa with inhibitor (see legend to Fig 7). After this time interval, factor IX activation in each of these mixtures was determined by the addition of factor IX so as to result in a final concentration of 100 nmol/L. Final concentrations of factor XIa and platelet supernatants reached are indicated in the table and were fivefold less in the diluted mixtures. The rate of factor IXa formation was then determined by withdrawing samples from these reaction mixtures at two and four minutes and assaying for factor IXa as described in Materials and Methods.

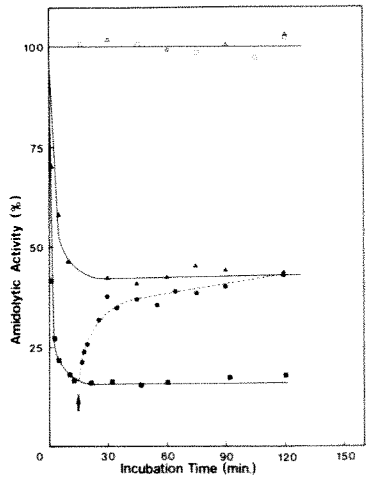

Fig 7. Reversibility of factor XIa inhibition. The supernatant (7.65 mL) of 2.6 × 10^8/mL platelets (obtained as described in the legend to Fig 5 was incubated for five minutes at 37 °C, and 0.9 mL factor XIa (4 nmol/L) was added. Immediately thereafter, 1.5 mL was subsampled and diluted fivefold in HEPES buffer containing 0.5 mg/mL human serum albumin. The time course of inhibition in each reaction mixture was followed by subsampling 475 μL to a cuvette at the time intervals indicated in the figure. Twenty-five microliters of S2366 (5.8 mmol/L) were added, and the rate of change of absorbance at 405 to 500 nm was determined at 37 °C on a DW2A Spectrophotometer (Amicon, Lexington, Mass). As a control, the amidolytic activity was followed over time in two reaction mixtures (prepared the same way) without supernatant present. The final concentrations in each reaction mixture were 0.5 mg/mL human serum albumin in HEPES buffer (pH 7.5): factor XIa (0.4 nmol/L), no platelet supernatant (□); factor XIa (0.4 nmol/L) and the supernatant of 2.2 × 10^8/mL platelets (■); factor XIa (0.08 nmol/L), no platelet supernatant (△); and factor XIa (0.08 nmol/L) and the supernatant of 4.4 × 10^7/mL platelets (▲). After equilibrium was reached, reversibility of the inhibition was verified by diluting part of the mixture containing the high concentrations of reactants fivefold and following the reappearance of amidolytic activity (●). The amidolytic activities determined for each reaction mixture in the presence of platelet supernatant were expressed as a percentage of the activities determined in the reaction mixture containing corresponding amounts of factor XIa but without supernatant present (open symbols).

Figure 8 shows Lineweaver-Burk plots of factor IX activation by factor XIa in the presence of varying amounts of platelet supernatant. These data show that both V_{max} and K_m were affected, indicating that the inhibitor is of the mixed type.

DISCUSSION

In this paper we report the effects of stimulated human platelets on the activation of human factor IX by factor XIa. We developed an assay that measures functional factor IXa via its ability to activate bovine factor X as described earlier[14] except that apart from phospholipids and $CaCl_2$ bovine factor VIIIa was also present in the assay. The

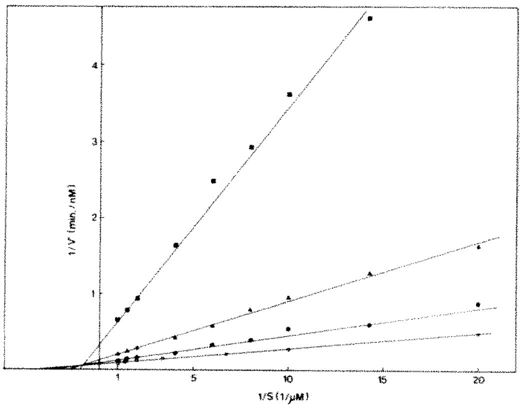

Fig 8. Lineweaver-Burk plots of factor IX activation by factor XIa at various inhibitor concentrations. The kinetic parameters (K_m for factor IX and V_{max}) were determined in the presence of varying amounts of inhibitor. Variation in the inhibitor concentrations was obtained by stimulating different platelet concentrations for twelve minutes at 37 °C in the presence of 3 mmol/L $CaCl_2$ with thrombin (1.2 nmol/L) and collagen (10 μg/mL) under continuous stirring followed by centrifugation for two minutes in an Eppendorf Microfuge to obtain the supernatants. Subsequently, the $CaCl_2$ concentration was adjusted to 10 mmol/L, and factor XIa was added and incubated for ten minutes at 37 °C before the addition of factor IX. The final reaction mixtures contained 10 mmol/L $CaCl_2$, 0.5 mg/mL human serum albumin, varying amounts of factor IX, and 0.1 nmol/L factor XIa in HEPES buffer (pH 7.5) without inhibitor (O) and in the presence of supernatant obtained from 2×10^7/mL platelets (●), 6×10^7/ml platelets (▲), and 2×10^8/mL platelets (■). Rates of factor IX activation were determined as described in Materials and Methods, and from these, the Lineweaver-Burk plots were constructed. The kinetic parameters obtained were 14.4 nmol/L factor IXa/min and 0.3 μmol/L (O), 10.0 nmol/L factor IXa/min and 0.37 μmol/L (●), 7.9 nmol/L factor IXa/min and 0.6 μmol/L (▲), and 3.0 nmol/L factor IXa/min and 0.99 μmol/L (■).

presence of factor VIIIa increases the sensitivity some 3,000-fold but has the disadvantage that for each new batch of factor VIII a new standard curve must be made. When this is done, a good day-to-day reproducibility can be achieved, and the assay allows accurate determination of factor IXa down to levels of 2.5 pmol/mL (Fig 1). Different batches of factor IXa, factor X, and phospholipid can be used interchangeably to produce the same standard curve provided equal amounts of these reagents are used. Using this assay, we determined the kinetic parameters of factor IX activation by factor XIa, and we found a K_m for factor IX of 0.3 μmol/L and a k_{cat} of 2.4 s^{-1}/factor XIa molecule of 160,000 mol wt. Some disagreement exists in the literature concerning these kinetic parameters. Walsh et al[10] using factor XIa activated with bovine factor XIIa found a K_m of 0.49 μmol/L and a k_{cat} of 7.7 s^{-1}, whereas Bajaj[13] using a trypsin-activated factor XIa found a k_m of 2.0 μmol/L and a k_{cat} of 0.34 s^{-1} (per 160,000 mol wt factor XIa). Both these studies made use of the tritiated activation peptide release. The kinetic parameters determined by us are rather close to the data reported by Walsh et al.[10]

Using this assay, we determined the effect of platelets on the rate of factor IXa formation by factor XIa. The data reported here clearly demonstrate that stimulated platelets cause a dramatic decrease in the rate of factor IX activation by factor XIa, whereas unstimulated platelets have no effect. This inhibition is caused by a direct interaction with factor XIa, since steady-state rates of factor IXa formation can only be achieved by incubation of factor XIa with the stimulated platelets, whereas preincubation of factor IX has no effect (Fig 2). Moreover, factor XIa amidolytic activity towards the chromogenic substrate S2366 is also inhibited (see Figs 6 and 7). Various platelet agonists can bring about this effect, the most potent being thrombin or the ionophore A23187. The set of agonists that can induce this effect (Table 1) as well as the rapid time course of the appearance of inhibitory activity after stimulation of the platelets (Fig 5) suggests the involvement of a release reaction. This is in accordance with the finding that the inhibitory activity is quantitatively recovered in the supernatant after centrifugation in a microfuge for five minutes (Fig 3). The amount of inhibitory activity is dependent on the amounts of stimulated platelets present. Thus, under the conditions used in our experiments, factor IX activation is not affected by the presence of 10^6/mL platelets. At the physiologic platelet concentration (2×10^8/mL), about 10% of the factor IX activation rate is left. Thus, the inhibitory activity reported here may play a role under physiologic circumstances. However, more experiments will be needed to gain insight into this.

The high apparent mol wt with which the inhibitor elutes from an ACA 22 column as well as the rather broad elution profile (see Fig 6) suggests that the inhibitor consists or is part of high-mol wt complexes. This may indicate that the inhibitor is associated with the so-called platelet dust.[33] However, an essential part of the large inhibitor molecules eluting from the ACA 22 column must be proteinlike in nature, since the inhibitory activity was completely destroyed after tryptic digestion. Moreover, the activity is also destroyed upon boiling. Therefore, at this moment we work under the hypothesis that the inhibitory activity is due to a protein that is released. This is most likely an antiprotease that reversibly blocks the factor XIa active sites both for factor IX activation and for S2366 conversion, since the inhibition seems reversible (see Table 3 and Fig 6). This inhibition is of the mixed type as determined by Lineweaver-Burk analysis of factor IX activation by factor XIa in the presence of varying amounts of inhibitor (Fig 8).

The main plasma inhibitors of factor XIa are α_1-antitrypsin and antithrombin III.[34] α_1-Antitrypsin is also present in platelets and can be secreted upon stimulation.[35] However, these inhibitors have a much lower molecular weight and irreversibly block factor XIa. Therefore, our data do not suggest any involvement of these proteins in the inhibition that we observe in our experiments. At this moment we do not know whether under different experimental conditions the α_1-antitrypsin present in platelets may become involved in the inhibition of factor IX activation by factor XIa.

It has been suggested that high-mol wt kininogen (HMWK) protects factor XIa against inactivation by plasma inhibitors,[34] and a recent report of Schmaier et al shows that HMWK can be secreted from human platelets upon stimulation.[36] The results presented here show that despite these amounts of HMWK present in the platelets the rate of factor IX activation by factor XIa decreases in the presence of stimulated platelets. Sinha et al have recently presented evidence that factor XIa needs HMWK to bind to stimulated platelets and that the amount of HMWK secreted by platelets is not sufficient to cause measureable factor XIa binding to triggered platelets.[37] Therefore, it will be important to investigate whether factor XIa binding via HMWK to stimulated platelets can prevent the inhibition of factor IXa formation caused by stimulated platelets in the absence of external HMWK.

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A New von Willebrand Variant (Type I, New York): Increased Ristocetin-Induced Platelet Aggregation and Plasma von Willebrand Factor Containing the Full Range of Multimers

By Harvey J. Weiss and Ira I. Sussman

We report three members of a family who had reduced levels of plasma von Willebrand factor (vWF) and increased ristocetin-induced platelet aggregation (RIPA) (aggregation of platelet-rich plasma with ristocetin at a concentration of 0.45 mg/mL), as previously reported in type IIB and pseudo–von Willebrand's disease (vWD). However, in contrast to the latter two disorders in which the larger vWF multimers are absent in plasma, the entire range of vWF multimers was observed in the patients' plasma after sodium dodecyl sulfate–agarose gel electrophoresis, and all vWF multimers (including the largest) were present in the same proportion as in normal plasma and type I vWD. Thus, despite increased RIPA, the levels and multimeric pattern of vWF in this family's plasma were indistinguishable from those in type I vWD in which RIPA is usually decreased. Addition of ristocetin to the patients' platelet-rich plasma resulted in the removal of vWF (and, more selectively, of the large multimers) at lower concentrations of ristocetin than normal, as in type IIB and pseudo-vWD. The defect in the patients was localized to their vWF, which had an enhanced capacity for aggregating washed normal platelets in the presence of low concentrations of ristocetin and for aggregating pseudo-vWD platelets (in the absence of ristocetin). Both glycoproteins (GP) Ib and IIb-IIIa were involved in the enhanced aggregation response. RIPA (at low ristocetin concentrations) in the patients' platelet-rich plasma was abolished by a monoclonal antibody (AP1) to GPIb and was markedly reduced by monoclonal antibodies (10E5 and LJP9) that block adenosine diphosphate and thrombin-induced binding of vWF and fibrinogen to GPIIb-IIIa but was unaffected by an antibody (LJP5) that only blocks vWF binding. Partial inhibition of the initial aggregation slope (and complete inhibition of second phase aggregation) was achieved with creatine phosphate/creatine phosphokinase. EDTA blocked second-phase aggregation but was without effect on the initial slope. The findings in this family combine some features of both type I vWD (normal pattern of vWF multimers in plasma) and type IIB vWD (increased RIPA) and further demonstrate the increasing complexity of the structure-function relationships in vWD.

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VON WILLEBRAND FACTOR (vWF) protein circulates in plasma as a series of multimers ranging in molecular weight (mol wt) from about 1 to 20 × 10^6 daltons.[1-5] Among its properties is the ability to bind to platelet glycoprotein (GP) Ib in the presence of ristocetin,[6-8] resulting in platelet aggregation.[9,10] One report has suggested that the larger multimers bind to GPIb with the highest affinity[11] and would therefore be the most effective in inducing ristocetin-induced platelet aggregation (RIPA), whereas others have found that the vWF monomer is comparable to that of the native protein.[12,13] The various plasma abnormalities in vWF that have been observed in patients with von Willebrand's disease (vWD) include a virtual absence of the protein (severe, type III vWD), a reduction in vWF but with all multimers present (type I vWD), or a selective absence of the larger multimers (type II vWD).[4,5,14] In type I and III vWD, RIPA is variably decreased in proportion to the reduction in plasma vWF,[10,15] and in most subtypes of type II vWD (such as IIA) in proportion to the decrease in the larger multimers.[16-19] However, a subtype of type II vWD has been described (type IIB) in which RIPA is increased despite the absence of the larger vWF multimers from the plasma.[4,5,19,20] In this disorder, platelet aggregation is observed with concentrations of ristocetin (0.3 to 0.6 mg/mL) that do not ordinarily produce aggregation in normal subjects.[19] It has been shown that the vWF in type IIB vWD binds more efficiently to platelets in the presence of low concentrations of ristocetin than does normal vWF[20] and that the larger multimers in these patients are also cleared more rapidly from plasma than in normal subjects.[21] Increased RIPA, associated with a loss of larger vWF multimers in plasma, is also characteristic of another disorder, pseudo-vWD[22] or platelet-type vWD,[23-25] in which the basic defect is not in the vWF protein but rather in the platelets, which have an enhanced capacity to adsorb vWF in the presence of low concentrations of ristocetin. The platelets of patients with pseudo-vWD were shown initially to aggregate directly with human vWF without the requirement for ristocetin,[22] and this was subsequently described in patients with platelet-type vWD as well.[24,25] In all likelihood, pseudo-vWD and platelet-type vWD are similar, if not identical, disorders.

We report herein a family with increased RIPA who do not appear to have any of the aforementioned variants of vWD. Unlike patients with type IIB vWD, the full range of vWF multimers (as in type I vWD) are present in their plasma. The increased RIPA was found to be due to an abnormal vWF rather than a platelet defect. Hence they do not have pseudo- (or platelet-type) vWD. They appear to have a previously undescribed subtype of vWD.

MATERIALS AND METHODS

Blood. Venous blood was mixed (9:1) with 3.2% sodium citrate and centrifuged to obtain platelet-rich plasma (PRP) and (platelet-poor) plasma as previously described.[10,15]

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Bleeding time. The bleeding time was determined with a Simplate-II bleeding time device (General Diagnostics, Morris Plains, NJ).

Standard hematologic studies on plasma. Prothrombin time and partial thromboplastin time (PTT) were determined by standard techniques, and specific coagulation factors (factors VIII and XI) were assayed with reference to pooled normal plasma by a one-stage technique[15] using congenitally deficient plasmas as substrates.[15] vWF antigen (previously designated FVIIIR:Ag) was assayed by the Laurell electroimmunoassay (EIA) as previously described.[18] Ristocetin cofactor in diluted plasma was assayed using washed normal platelets and a ristocetin concentration of 1.25 mg/mL.[15]

Platelet studies. Platelets were counted by phase microscopy. Platelet aggregation induced in PRP by collagen, adenosine diphosphate (ADP), and epinephrine was studied in the aggregometer (Payton Associates, Buffalo) as previously described.[15] Aggregation by graded concentrations of ristocetin was determined by adding 20 μL of ristocetin (Lundbeck, Copenhagen) to 1.0 mL of PRP to achieve a final concentration ranging from 0.3 to 1.5 mg/mL. Results obtained were expressed as the initial slope of the aggregation curve (normalized to a chart speed of 20 mm/min) and as the maximal degree of aggregation.[15] vWF antigen in platelets was measured on lysates of washed platelets by EIA as previously described.[18,22] Platelet adhesion to rabbit subendothelium was measured by exposing deendothelialized segments of rabbit aorta to citrated whole blood[26] at a shear rate of 2,600 s^{-1} for five minutes.[27]

Special studies of vWF. Special studies on patient and normal vWF were performed using either plasma or various plasma fractions. Cryoprecipitate was prepared by thawing frozen (−70 °C) citrated plasma (prepared as previously described) for 16 hours at 4 °C.[22] Further separation of vWF was obtained by agarose gel chromatography. Cryoprecipitate, 1.7 to 2 mL, was applied to a 2½ × 30-cm column of agarose A-15m, and elution performed with 0.05 mol/L Tris-0.15 mol/L NaCl buffer, pH 7.1, as previously described.[28] vWF multimers were identified by electrophoresis of plasma or platelet lysates in 0.1% sodium dodecyl sulfate (SDS)–1.5% glyoxyl agarose according to the method of Hoyer and Shainoff.[1] Gels, 10 × 18 × 0.2 cm, were cast on gel bond film; 20-μL samples were electrophoresed to a length of 7 cm at a constant voltage of 100 V. The vWF multimers were visualized by autoradiography following incubation with ^{125}I-labeled rabbit antihuman vWF (RAH vWF). RAH vWF[28] was iodinated according to the iodogen method[29] and was purified on a 1 × 9-cm Sepharose 4B, vWF-affinity column that was eluted with 0.1 mol/L glycine, 0.5 mol/L NaCl, pH 2.8. Purification of vWF was performed as previously described.[28] The percentage of the total vWF represented by individual multimers was determined by densitometry. Scans of each gel were performed on a Quick Scan Jr (Helena Laboratories, Beamont, Tex) and the area under each curve determined by integration. The first six bands could be individually determined, whereas the areas under the remaining bands were grouped together. Each band was expressed as a percentage of the total area. Crossed immunoelectrophoresis was performed on 0.9%-agarose plates. Thirty-microliter plasma samples were electrophoresed at 200 V constant voltage in the first direction and 15 mA (two plates) constant current in the second direction. Precipitant arcs were visualized with either Coomassie blue dye or by autoradiography. In the latter, 100,000 cpm ^{125}I RAH vWF was added to the agarose gel in the second phase of the electrophoresis. The sialic acid content of highly purified vWF was determined by Dr Sandor Shapiro (Jefferson College, Philadelphia), as previously described,[30] from four to six units of plasma (1,000 to 1,500 mL) obtained by plasmapheresis, the latter kindly performed by Dr Randy Levine (St Luke's-Roosevelt Hospital Center). The ability of vWF in either plasma, cryoprecipitate, or vWF fractions to support aggregation of washed platelets was studied using platelets (from normal subjects) that had been separated from citrated PRP by albumin density gradient separation[22] and suspended in calcium-free Tyrode's buffer (pH 7.3) at a final platelet count of 300,000 to 1,200,000/μL as previously described.[22] Adsorption of plasma vWF to platelets in the presence of ristocetin was performed as previously described.[22] For these studies, a suspension of washed platelets was incubated with an equal volume of plasma in the presence of various concentrations of ristocetin for 30 minutes at 37 °C, and after centrifugation at 2000 g for ten minutes, the supernatant plasmas were frozen at −70 °C until studied. The vWF antigen in the supernatant was assayed by radioimmunoassay,[31] (kindly performed by Dr Leon Hoyer, University of Connecticut Health Science Center, Farmington) and vWF multimers were determined as described previously.

Platelet inhibitors. The inhibitory effects of several monoclonal antibodies to platelet GP on RIPA was studied by the addition of these antibodies to PRP. A previously described[32] monoclonal antibody (AP1) against platelet GPIb that blocks ristocetin-induced binding of vWF to platelets[32] was kindly provided by Drs Robert Montgomery and Thomas Kunicki (Blood Center of Southeastern Wisconsin, Milwaukee). A monoclonal antibody (10E5) against GPIIb-IIIa was a gift from Dr Barry Coller (State University of New York, Stony Brook). This antibody has been previously shown to block the binding of fibrinogen to thrombin and ADP (but not ristocetin)-stimulated platelets[33] and, more recently, has been shown to block the binding of vWF, fibronectin, and thrombospondin as well.[34] Several other monovalent Fab fragments of monoclonal antibodies against GPIIb-IIIa were a gift of Dr Zaverio Ruggeri (Scripps Clinic and Research Foundation, La Jolla, Calif). Antibody LJP9 inhibits the thrombin and ADP-induced binding to GPIIb-IIIa of both vWF and fibrinogen.[35] Antibody LJP5 inhibits the binding of vWF but not fibrinogen,[35] and antibody LJP10 binds to BPIIb-IIIa without inhibiting uptake of either vWF or fibrinogen (Dr Z. Ruggeri, personal communication). A possible role for ADP was studied using PRP prepared from blood anticoagulated (9:1) with 3.2% sodium citrate containing 90 mmol/L creatine phosphate and 60 U/mL creatine phosphokinase (CP/CPK) (Sigma Chemical Co, St Louis). The effect of adding Na_2 EDTA was also studied. The studies using CP/CPK, EDTA, and monoclonal antibodies 10E5 and LJP9 were performed at concentrations of these substances that completely abolished platelet aggregation by 20 μmol/L ADP.

Patients. Three members of a family with a mild bleeding history were studied. Patient 1, the propositus, is a 24-year-old male who reported a lifelong history of easy bruising. He bled excessively after a tonsillectomy at age 2½ and received plasma transfusions as treatment for a trauma-induced hematoma in his leg at age 13. However, he did not bleed excessively after a herniorrhaphy at age 1½ yrs. His twin sister (patient 2) also bruises easily, bled excessively after a tonsillectomy at age 2½, and at age 16 received blood transfusions for treatment of a large, painful hematoma on the buttocks. A second sister, aged 29, was not studied but is said to bruise easily and had life-threatening hemorrhage after a tonsillectomy at age 4. In addition, she suffered a hemarthrosis of the right ankle at age 23 (for which she received cryoprecipitate) and, in the same year, was treated with fresh-frozen plasma for about ten days after right knee surgery complicated by bleeding and the development of an anterior compartment syndrome. The 48-year-old mother of the propositus (patient 3) bled extensively after tooth extractions and postpartum, had one episode of unexplained gastrointestinal bleeding, and has received both plasma and cryoprecipitate for treatment of bleeding episodes. Patient 1 was studied by us on multiple occasions, and patient 2 was studied twice. Samples of frozen plasma on patient 3 (in whom variably depressed, but sometimes normal, levels of vWF and factor XI have been found elsewhere) were kindly provided in June 1985 by Dr Judith Ratzan of Miami. The second affected sister of the propositus was not studied but has been diagnosed elsewhere as having vWD (with a normal factor XI level).

Other subjects. Control subjects were normal hospital personnel, aged 21 to 50. Patients with type I vWD included those with decreased levels of vWF in plasma and platelets, which we have previously designated as type I-1 vWD, and those with decreased plasma but normal platelet vWF levels (type I-2 vWD[18]).

RESULTS

Basic hematologic studies. Patients 1 and 2 were studied in our laboratory, and selected studies on patient 3 were done on samples of frozen plasma. Normal values were obtained for hemoglobin, white blood count, prothrombin time, and platelet count. Other studies are shown in Table 1. The bleeding time (performed once) was within the range of normal values. Factor VIII:C and vWF values on the propositus (patient 1) were obtained on four separate occasions. The average value obtained for Factor VIII:C was 26 U/dL; for vWF antigen, 38 U/dL; and for ristocetin cofactor, 41 U/dL. The relatively concordant decrease in these values is characteristic of the findings in type I vWD. The values of factor VIII:C, vWF antigen, and ristocetin cofactor obtained in his twin sister (patient 2) and mother (patient 3) were, in most cases, just at or somewhat below the lower limits of the control values (Table 1). The overall findings in the three affected family members studied suggest a diagnosis of type I vWD with variable degrees of penetrance. Since the vWF antigen in their platelets was normal (Table 1), they have the subtype of von Willebrand's disease that we have previously designated as type I-2.[18] In addition to the aforementioned findings, factor XI was moderately decreased in patients 1 and 3 (40 and 34 U/dL, respectively) and was at the lower limit of normal in patient 2 (60 U/dL).

Table 1. Laboratory Studies

	Control Values	Patient 1	Patient 2	Patient 3*
Bleeding time (min)	<9	4	7	—
Plasma values (U/dL)				
Factor VIII: C	50–150	26	51	51
vWF antigen (EIA)	55–160	38	58	71
Ristocetin cofactor	50–150	41	46	50
Factor XI	60–150	40	60	34
Platelets				
Number (× 10^{-3})/μL	180–350	278	232	normal†
vWF antigen (U/10^9)	0.25–0.69	0.46	0.39	—
Ristocetin aggregation‡				
0.3 mg/mL	0	0	0	—
0.45 mg/mL	0	60	90	—
0.6 mg/mL	0	91	85	—
0.9 mg/mL	0–88	93	100	—
1.2 mg/mL	5–84	99	100	—

*Assayed on frozen plasmas. (See Note Added in Proof).

†Performed elsewhere.

‡Control values obtained on eight to 13 normal subjects.

In patients 1 and 2, average results shown for plasma values and ristocetin aggregation are for studies obtained on four and two separate days, respectively.

RIPA. On all occasions studied, the addition of ristocetin to the PRP of both patients 1 (four occasions) and 2 (two occasions) resulted in platelet aggregation (60% to 91%) at concentrations (0.45 and 0.60 mg/mL) that never produced aggregation in normal subjects (Fig 1 and Table 1). This enhanced response to ristocetin is typical of previous findings in type IIB[4,5,19,20] and pseudo-[22] or platelet-type vWD.[23-25] No aggregation resulted upon the addition of either cryoprecipitate or vWF fractions to patient PRP (data not shown), nor was spontaneous aggregation observed.

Multimeric structure of vWF. Figure 2 shows the multimer pattern of vWF in representative plasma samples for the three family members (patients 1, 2, and 3), three control subjects (N1, N2 [father of the propositus], N3), and three patients with typical type I vWD (two with type I-1 and one with type I-2). The full range of vWF multimers was consistently present in all three patients; their multimer patterns were indistinguishable from those in control plasmas or in patients with type I vWD. These patterns were observed in plasma samples obtained from patient 1 on four different days at intervals of 4, 2, and 2 months, from patient 2 on two samples obtained 1 week apart, and on one sample of plasma from patient 3. The conclusions drawn from visual examination of the gels were confirmed by densitometric scan in that the percentage of the total vWF contributed by the larger multimers (those of mol wt greater than band 6), as well as by all other multimers, were the same in the three affected family members as in the controls or type I subjects (Table 2). The pattern of vWF multimers in their platelets was also normal (data not shown).

Enhanced capacity of patient vWF to support ristocetin aggregation. Several types of studies were done that demonstrated that the enhanced RIPA in the affected family members was due to an abnormality in their plasma vWF. In one group of studies, platelet aggregation induced by increasing concentrations of ristocetin was assessed in a mixture containing washed normal platelets and either cryoprecipitate (at a final vWF antigen concentration of 35 U/dL) or

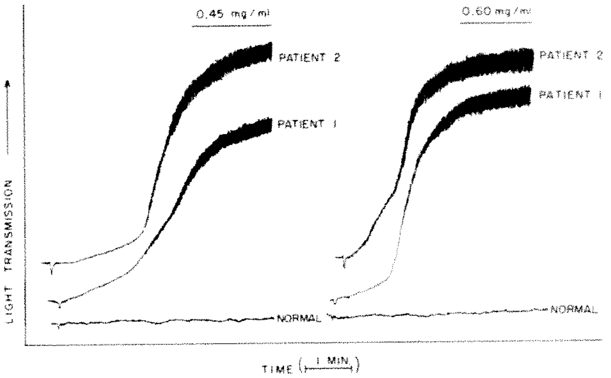

Fig 1. **Aggregation of patients' PRP by low concentration of ristocetin. Ristocetin was added to citrated PRP in a concentration of 0.45 or 0.60 mg/mL. Results are shown for patients 1, 2, and a typical normal subject. For complete dose-response data, see Table 1.**

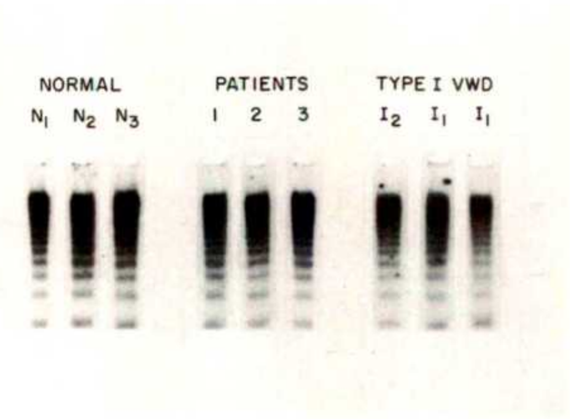

Fig 2. Multimeric composition of plasma vWF. Autoradiographic pattern of plasma vWF multimers detected by ^{125}I-labeled anti-vWF after electrophoresis in SDS–glyoxyl agarose. The anode is at the bottom. Studies are shown for patients 1, 2, 3, for three normal subjects (N1, N3, and N2, the father of patients 1 and 2), and for three patients with type I (two with I-1 and one with I-2) vWD. The percentage of the total vWF for each of the first six multimers and for the multimers of mol wt greater than band 6 is shown in Table 2.

plasma (at a final vWF antigen concentration of 20 U/dL). The enhanced capacity of cryoprecipitate from patient 1 to support RIPA at low concentrations of ristocetin is shown in Fig 3A and 3B. Similar studies using plasma are shown in Fig 3C and demonstrate the enhanced capacity of vWF from patients 1, 2, and 3 to support RIPA when compared with plasmas from normal subjects or from patients with either type I-1 or type I-2 vWD. The experiment was modified by using, instead of a fixed concentration of vWF, increasing concentrations of vWF and a fixed, low concentration (0.6 mg/mL) of ristocetin. The enhanced capacity of vWF from patient 1 to support platelet aggregation by a low concentration of ristocetin is shown in Fig 3D. Finally, the increased reactivity of patient vWF with platelets in the absence of ristocetin was demonstrated using PRP from a subject with pseudo-vWD. We have previously shown that platelets from these patients are aggregated by human vWF without a requirement for ristocetin.[22] The enhanced capacity of vWF from patients 1 and 2 to induce platelet aggregation in a patient with pseudo-vWF is shown in Fig 4.

Table 2. Densitometric Scan of vWF Multimers in Plasma

	Percentage of Total vWF*								
	Normal			Patients			Type I vWD		
Multimer No.	N1	N2	N3	1	2	3	I-2	I-1	I-1
>6	60	53	58	56	55	57	56	55	58
6	6	8	7	7	7	6	6	8	6
5	7	8	6	8	8	7	7	6	6
4	7	9	9	9	8	8	8	9	7
3	6	7	6	7	8	9	7	7	6
2	6	8	7	7	8	6	7	7	7
1	6	7	7	7	7	7	9	9	10

*The percentage of the total vWF for bands 1 (the multimer of lowest mol wt) through band 6 and for the vWF accounting for all multimers with mol wt > band 6, determined by densitometric scan of the gels shown in Fig 2.

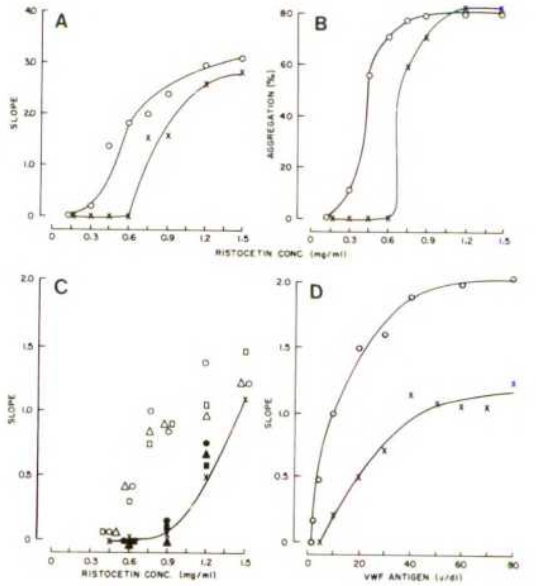

Fig 3. Capacity of vWF to support ristocetin-induced aggregation of washed normal platelets. Platelet aggregation induced by 10 μL of ristocetin was studied in a mixture containing a suspension of albumin density gradient, washed normal platelets and plasma, cryoprecipitate, or fractions prepared from the latter. (1) Top: initial slope (A) or aggregation (B) in a mixture containing 240 μL platelet suspension + 250 μL cryoprecipitate (in Tris-saline, pH 7.3, to a vWF concentration of 70 U/dL) from patient 1 (○) or a control (X) and increasing concentrations of ristocetin. (2) Bottom left (C): 240 μL platelet suspension + 250 μL of pooled normal or patient plasma containing 0.1 units of vWF antigen (achieved, where necessary, by dilution with type III vWD plasma) plus increasing concentrations of ristocetin. The initial slope is shown for normal subjects (X); patients 1 (○), 2 (△), and 3 (□); and patients with type I-1 (●, ■) and I-2 (▲) vWD. (3) Bottom right (D): 140 μL washed platelets and 350 μL of a vWF fraction diluted in Tris-saline to contain variable amounts of vWF + ristocetin (final concentration 0.6 mg/mL). The initial slopes obtained with increasing (final) concentrations of vWF antigen are shown for patient 1 (○) and a normal control (X).

Increased adsorption to platelets of vWF and of the larger vWF multimers from patient plasma in the presence of ristocetin. In these studies, increasing concentrations of ristocetin were incubated at 37 °C. for 30 minutes with a mixture containing equal volumes of washed normal platelets and plasma from either a normal subject or patient 1. The final vWF antigen concentration in the mixture without ristocetin was 20 U/dL, obtained in the case of the normal subject by prior dilution with plasma from a patient with severe (type III) vWD. After centrifugation, the vWF antigen content in the supernatant was measured by radioimmunoassay, and the percentage of vWF antigen remaining (not adsorbed to platelets) was calculated with reference to a control incubation mixture. In addition, the multimer pattern of vWF in each supernatant sample was determined. Figure 5 demonstrates the enhanced ristocetin-induced adsorption of vWF and the larger vWF multimers observed with the plasma of patient 1. In contrast to this abnormality of plasma vWF, the capacity of the patient's platelets to adsorb normal vWF with increasing ristocetin concentrations was the same as in normal platelets (data not shown).

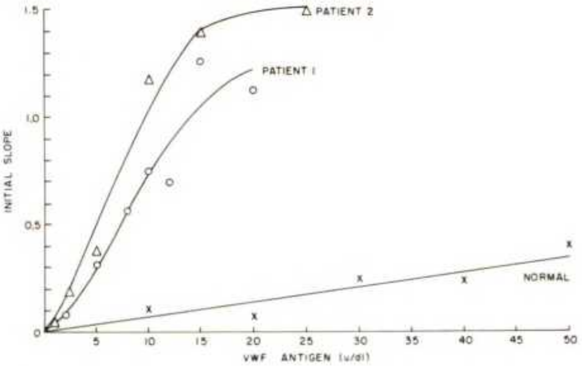

Fig 4. **Aggregation of platelets of a patient with pseudo-vWD by plasma. Plasma from patients 1, 2, or a normal control were mixed, when necessary, with plasma from a patient with type III (severe) vWD to achieve varying concentrations of vWF antigen, and 0.5 mL was added to an aggregometer cuvette containing 0.5 mL of PRP from a patient with pseudo-vWD[22] whose platelets are aggregated directly by vWF without a requirement for ristocetin. The figure shows the initial slope of the aggregation tracings.**

Sialic content of vWF. The sialic content of highly purified vWF was the same (98.3 nmol/mg protein) as in a control subject studied in parallel (89.7 nmol/mg protein). Both of these values are somewhat lower than in previous normals studied in the laboratory of Dr Sandor Shapiro (131 ± 16 nmol/mg protein).[30] The reason for this is not clear but could be related to the fact that because of the limited sample only one, rather than two, cryoprecipitations were used in the present study and this could have resulted in a vWF preparation of somewhat lesser purity.

Crossed immunoelectrophoresis. The precipitin arc of vWF obtained by crossed immunoelectrophoresis of plasma from patient 1 was of the same configuration as that observed in the plasma from a normal subject or from a patient with type I vWD (data not shown).

Effect of platelet inhibitors and monoclonal antibodies to platelet GP on RIPA. PRP from patient 1 was used in the study, and the results are summarized in Fig 6. Platelet aggregation by 0.6 mg/mL ristocetin was completely blocked by a monoclonal antibody (API) to GPIb.[32] The initial slope of the aggregation tracing was markedly diminished by two different monoclonal antibodies (10E5 and LJP9) that block thrombin and ADP-induced binding of vWF and fibrinogen to GPIIb-IIIa.[33-35] In contrast, no inhibitory effects on RIPA were observed with either monoclonal antibody LJP5, which blocks thrombin-induced binding of vWF, but not fibrinogen, to GPIIb-IIIa,[35] or with antibody LJP10, which binds to GPIIb-IIIa but does not inhibit the binding or any known protein. The effects of several known platelet inhibitors were also studied. EDTA (0.1%) had no effect on (and may have even enhanced) the initial response (slope) to ristocetin, but it abolished secondary aggregation. The ADP-removing system CP/CPK inhibited the initial slope and abolished secondary aggregation.

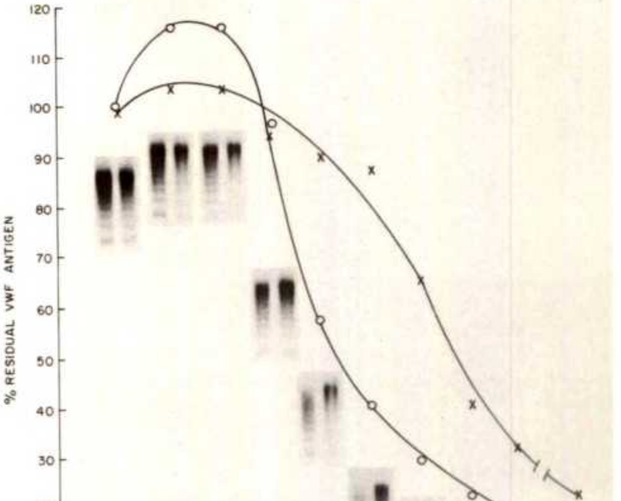

Fig 5. **Adsorption of plasma vWF to normal washed platelets in the presence of ristocetin. A suspension of washed normal platelets (500 μL) was incubated with an equal volume of plasma (vWF antigen content, 40 U/dL) from patient 1 (O) or a normal subject (X) and adjusted to the same vWF antigen concentration by dilution with plasma from a patient with severe (type III) vWD in the presence of increasing concentrations of ristocetin for 30 minutes at 37 °C without stirring. After centrifugation, the supernatant plasma was assayed for vWF antigen by radioimmunoassay, and the amount of vWF adsorbed to platelets was calculated with reference to a control specimen in which platelets and ristocetin were omitted (no platelets). The vWF multimer patterns shown for each supernatant (patient, left; control, right) demonstrate the more selective removal of the larger vWF multimers from the plasma of patient 1 with increasing concentrations of ristocetin.**

Platelet adhesion to subendothelium. Platelet adhesion to subendothelium, studied in citrated blood at a shear rate of 2,600 s^{-1}, was normal in both patients 1 and 2 (58% and 55% *v* control values of 42% ± 5%). To study specifically the properties of patient vWF in supporting adhesion, we added increasing concentrations of normal or patient vWF (in cryoprecipitate) to samples of citrated blood from a patient with severe (type III) vWD and then studied platelet adhesion to subendothelium. When compared at comparable levels of vWF antigen, the ability of patient vWF to correct the adhesion defect in type III vWD was, if anything, somewhat greater than that observed with normal vWF (data not shown).

DISCUSSION

Except for the enhanced platelet aggregation of their platelets by low concentrations of ristocetin, many of the findings in the family reported herein are those of type I vWD. Thus, factor VIII:C and vWF-related properties,

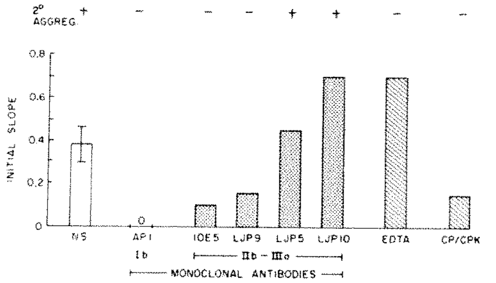

Fig 6. Effect of monoclonal antibodies to platelet GP and platelet inhibitors on platelet aggregation by a low concentration of ristocetin. To 940 μL of PRP from patient 1 was added the following substances and, where necessary, normal saline (NS) to a final volume of 980 μL and the final concentrations indicated: (1) 0 to 35 μL normal saline, n = 4; (2) 40 μL of a monoclonal antibody (AP1) to GPIb, 4 μg/mL, (3) monoclonal antibodies to GPIIb-IIIa as follows: 20 μL 10E5, 10 μg/mL; 5 μL LJP9, 50 μg/mL; 10 μL of LJP5, 50 μg/mL; and 20 μL of LJP10, 100 μg/mL; (4) 20 μL EDTA, 0.1%; or (5) 980 μL of PRP prepared from blood anticoagulated with sodium citrate containing CP/CPK (see Materials and Methods). After the addition of 20 μL of ristocetin (final concentration, 0.6 mg/mL), platelet aggregation was determined. The figure depicts the value of the initial aggregation slope (including the range for the four control samples) and the presence (+) or absence (−) of secondary aggregation following the initial aggregation response.

particularly in the propositus (patient 1), were decreased concordantly, and the complete range of vWF multimers was observed in their plasma (Fig 2). In addition, the proportion of the larger multimers to the total vWF was the same as in normal subjects and patients with typical type I vWD (Fig 2 and Table 2), thereby distinguishing this family from patients designated as type I B[36] in whom the larger multimers are present, but decreased in amounts relative to the other multimers. We have previously identified other subtypes of type I vWD according to the content of vWF antigen in plasma and platelets.[18] In the subtype designated I-1, the amount of vWF antigen in both plasma and platelets was decreased, whereas platelet vWF antigen was normal in type I-2. Findings entirely similar to these have recently been reported by Mannucci et al.[37] The family described herein had normal levels of vWF antigen in their platelets (Table 1) and hence has type I-2 vWD according to our nomenclature. Neither we nor others have ever observed enhanced RIPA in type I vWD, and specifically, we did not observe this in two recently studied unrelated patients with the type I-2 subtype. Previously, an increase in RIPA has been described in only two disorders, type IIB vWD[4,5,19,20] and pseudo-[22] or platelet-type vWD,[23,24,38,39] in both of which the larger vWF multimers are absent in plasma. Since the family reported herein has a type I plasma vWF multimer pattern, they cannot be classified as having either of these two disorders. However, as in type IIB-vWD, the defect was clearly localized to an abnormality in their vWF rather than in their platelets (as is the case in pseudo- or platelet-type vWD). Similar to the findings in type IIB vWD,[20] their vWF had a greater capacity for supporting platelet aggregation by low concentrations of ristocetin than vWF from normal subjects or from three patients with more typical type I vWD (Fig 3). In addition, patient vWF was more effective than normal vWF in aggregating the platelets of a patient with pseudo-vWD (Fig 4), demonstrating that the enhanced reactivity of their vWF towards platelets can be demonstrated in the absence of ristocetin. By analogy with type IIB, it might be appropriate to designate these patients as having type IB vWD, but since this term has already been used for another subtype of type I,[36] we have chosen to designate our family as having vWD type I–New York or, alternatively, type IV vWD.

The basis for the enhanced capacity of the vWF in patients with vWD type I–New York to support platelet aggregation by low concentrations of ristocetin remains to be determined. As in type IIB vWD,[19,20] this enhanced aggregation was associated with increased adsorption to platelets of vWF and, more specifically, of the larger vWF multimers (Fig 5), which may bind to GPIb with the highest affinity.[11] Not surprisingly, platelet aggregation by low concentrations of ristocetin was completely blocked by a monoclonal antibody to GPIb since aggregation by ristocetin requires, initially, the binding of vWF to this platelet membrane GP.[6–8,40–42] Aggregation by low concentrations of ristocetin was also greatly diminished by monoclonal antibodies to GPIIb-IIIa (10E5 and LJP9) that block ADP- and thrombin-induced binding of vWF and fibrinogen to this membrane GP,[33,35] but was not inhibited by antibody LJP5, which only blocks vWF binding.[35] These findings together with the inhibitory effect of the ADP-consuming system CP/CPK suggest that the binding to GPIIb-IIIa of fibrinogen (but not vWF) through endogenous platelet ADP may also contribute to the enhanced RIPA in vWD type I–New York. These conclusions are consistent with recent studies that have demonstrated that the binding of fibrinogen to GPIIb-IIIa plays a role in the aggregation of normal platelets that is initiated by binding of vWF to GPIb.[43,44] The enhanced initial response to low concentrations of ristocetin in the presence of EDTA (which blocks binding of proteins to GPIIb-IIIa[5]) may be related to the fact that calcium is not required for the initial binding of vWF to GPIb[8] and suggests that this binding may even be enhanced in vWD type I–New York.

Several aspects of this disorder remain to be clarified. For one, it is not clear why, as in type IIB vWD, the larger multimers are more selectively removed from plasma by low concentrations of ristocetin, yet (unlike type IIB) the native plasma contains the full range of multimers (and the same proportion of larger multimers as in normal plasma). In theory, an increased clearance of larger multimers from plasma, as in type IIB vWD,[21] could be compensated for by increased synthesis, but we have no evidence to support this possibility. The relationship of the abnormal vWF in vWD type I–New York to the bleeding disorder in this family is also not entirely clear. Platelet adhesion (in citrated blood) to subendothelium was not diminished in these patients under conditions that have demonstrated decreased adhesion in patients with severe vWD,[26] although we have not systematically studied patients with milder forms of type I vWD. However, we found no evidence to suggest that the vWF in type I–New York is deficient in its capacity to support platelet adhesion, and in fact, where this was specifically examined, vWF type I–New York appeared to correct the platelet adhesion defect in severe vWD to a greater extent than normal vWF. An interpretation of the findings in this study is further complicated by the somewhat decreased factor XI values in two members of the family, although this was normal in one other clinically affected family member (patient 2) and was said to be normal in a second severely affected sister of the propositus who was not studied by us. In all likelihood, the bleeding disorder in the family is probably the result of low to marginal levels of vWF and factor VIII:C, and in some cases, factor XI, which are unable to support normal hemostasis under some circumstances. The findings in the present study demonstrate further the evolving complexity of the clinical and laboratory findings in vWD and demonstrate the problems involved in attempting to define the disorder in terms of either the plasma multimer patterns or functional studies such as those involving ristocetin aggregation. The family designated as having vWD type I–New York in the present study has features of both type I vWD (multimer patterns) and type IIB vWD (enhanced ristocetin aggregation) and underscores the desirability of assigning, ultimately, subtypes on the basis of the molecular defects in the vWF protein.

NOTE ADDED IN PROOF

We recently studied patient 3 in our laboratory and, on freshly drawn blood specimens, obtained values as follows: platelet aggregation 61% at a ristocetin concentration of 0.6 mg/mL, Factor VIII: C = 64 U/dL, Factor XI = 65 U/dL, vWF antigen = 52 U/dL, and ristocetin cofactor = 50 U/dL. Platelets, 305,000/μL; bleeding time, 7 min.

ACKNOWLEDGMENT

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Nonrandom Distribution of Iron in Circulating Human Transferrin

By Olga Zak and Philip Aisen

By combining the urea gel electrophoresis technique of Makey and Seal with Western immunoblotting, a method has been developed for analyzing the distribution of iron between the two sites of circulating human transferrin. The new method avoids exposure of samples to a nonphysiologic pH that may promote removal or redistribution of iron from the protein; this facilitates examination of multiple samples at one time. Analysis of 21 freshly drawn specimens from normal human subjects confirms previous reports that iron is not randomly distributed in the specific sites of transferrin. Rather, there is a considerable range in the ratio of occupancies of N-terminal and C-terminal sites (N:C ratio), from 0.31 to 6.87 in the present study, with the N-terminal site predominantly occupied in most subjects. The N:C ratio correlates modestly with serum iron concentration ($r = .54$). Possible flaws in studies indicating a random occupancy of the specific sites of circulating transferrin may lie in the low pH to which samples may be exposed during procedures based on isoelectric focusing or in drawing inferences from data considering only total monoferric transferrin rather than the two distinguishable monoferric species.

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DIFFERENCES between the two iron-binding sites of human serum transferrin have been revealed by a wide variety of thermodynamic,[1-3] kinetic,[4] and spectroscopic[1,5-7] measurements. However, studies of the functional properties of the two distinguishable sites carried out in vitro in attempts to elucidate any physiologic roles of these differences have led to inconsistent results.[8-11] Clearly, if the sites are unequally populated in the circulation, then differences in their physiologic behavior in vivo must also exist. Previous investigations bearing on this question have led to conflicting findings. Methods using urea gel electrophoresis have generally shown that the sites are not equally populated,[12,13] the more weakly binding B-site in the N-terminal domain of the protein being predominantly occupied. Another study using isoelectric focusing in polyacrylamide gels to resolve the four possible species of transferrin of varying content (iron-free or apotransferrin, monoferric transferrin bearing its iron atom at the C-terminal or A-site, monoferric transferrin occupied at the N-terminal or B-site, and saturated or diferric transferrin) has also indicated that iron is nonrandomly distributed between the binding sites of circulating transferrin.[14] However, a recent report, also based on isoelectric focusing, has suggested that iron is randomly distributed, implying that the two sites are equally occupied whatever the level of iron saturation.[15] Because of the importance of the site occupancies in achieving understanding of the molecular physiology of iron transport, we have reexamined the problem using procedures designed to minimize the redistribution of iron in samples exposed to a nonphysiologic pH[1,13,16] or stored under conditions in which migration of iron may be favored.[13,16] Our present findings reinforce earlier inferences from studies in this laboratory[1] and by Williams' group.[13,16] Additionally, we report possible pitfalls in the collection and analysis of data in studies based on isoelectric focusing.[15]

MATERIALS AND METHODS

Transferrin

Human serum transferrin was isolated from a 40% to 75% ammonium sulfate fraction of blood bank plasma by chromatography with diethylaminoethyl (DEAE)–Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ) following previously detailed procedures.[1] Preparations were freed of iron, then brought to the desired degree of saturation by the addition of appropriate quantities of freshly prepared ferrous ammonium sulfate. The absorbancy at 280 nm of millimolar apotransferrin was taken as 86.6.

Reagents and Buffers

Reagent grade chemicals were used for all preparations. Buffers for electrophoresis were passed through a column of Chelex-100 (Bio-Rad Laboratories, Richmond, Calif) to minimize contamination with extraneous metal ions. Ampholyte (pH 5 to 8) was obtained as 40% Ampholine from LKB (Gaithersburg, Md), acrylamide and bisacrylamide were purchased from Eastman Kodak (Rochester, NY) and other reagents from standard laboratory suppliers. Doubly distilled, deionized water was used in all preparations, and glassware was acid washed.

Collection of Blood Specimens

Samples of venous blood from normal human volunteers were collected in acid-washed tubes and allowed to clot for no more than 1½ hours under Parafilm caps at room temperature before removal of serum. After this time the pH of the serum differed by less than 0.01 units from the pH of serum obtained from preparations clotted in a 5% CO_2 incubator. Because we have verified the observations of Williams and Moreton that storage of serum at ordinary freezer temperature promotes redistribution of iron to the C-terminal site of transferrin,[13] all samples were analyzed immediately after collection.

Electrophoretic Methods

The classical urea gel electrophoresis technique of Makey and Seal,[17] widely adopted for studies of iron occupancy of the two sites of transferrin,[18] has been modified for the present work. Because of the finding that higher than physiologic pH favors binding of iron at the N-terminal site of transferrin,[16] we have substituted a 0.089 mol/L Tris-acetate buffer at pH 7.4 for the pH-8.4 Tris-borate buffer in the original Makey-Seal procedure. Electrophoresis was initiated at an electric field of 20 V/cm, gradually increased to 30 V/cm, then continued for a total of 550 V h/cm. This resulted in separation of the four molecular species of transferrin with resolution comparable to that obtained with the original Makey-Seal procedure.

Gels of purified transferrin preparations were stained with Coo-

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massie blue following standard procedures. For analysis of serum samples, autoradiograms of Western blot preparations were made using the Bio-Rad Trans-Blot cell, largely following the methods of Burnette.[19] Urea gels were washed twice, for ten minutes each time, with ice-cold 0.02 mol/L barbital buffer, pH 8.6, then three times with transfer buffer consisting of 0.192 mol/L glycine, 0.025 mol/L Tris, and 10% methanol at pH 8.8. Electrophoretic transfer of proteins from gel to nitrocellulose paper was carried out in a cold room for 1½ hours at 100 V followed by 2½ hours at 50 V. Nitrocellulose paper bearing the transferred protein was washed for one hour in a buffer of 0.9% NaCl, 0.01 mol/L Tris, 0.1% bovine serum albumin, and 0.05% Tween 20, then for an additional four hours (or overnight) in a buffer containing 1% bovine serum albumin but no detergent. To display transferrin bands, autoradiography was carried out with nitrocellulose sheets that had been incubated with affinity-purified, ^{125}I-labeled rabbit antibody to human transferrin. This antibody gave single lines of identity between purified transferrin and human serum by the double-diffusion technique of Ouchterlony. The Western blot method proved simpler and more readily applicable to the study of multiple serum specimens (Fig 1A) than the previously used crossed immunoelectrophoresis procedure.[12]

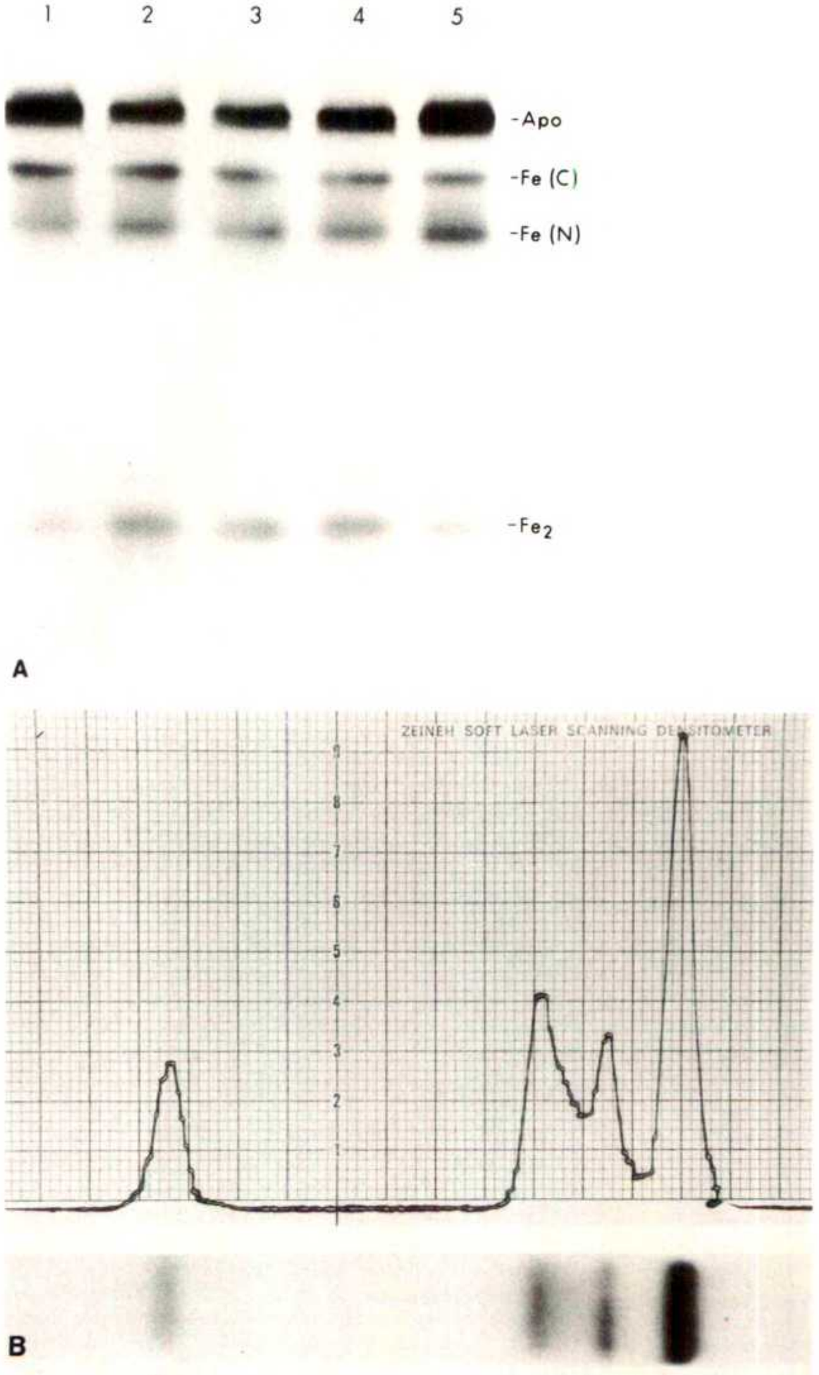

Fig 1. Analysis of transferrin species in human serum by Western blotting. (A) Five different serum samples with N:C ratios of 0.79 (lane 1), 1.08 (lane 2), 1.32 (lane 3), 1.27 (lane 4) and 2.24 (lane 5). (B) Densitometric scan of a serum sample showing an N:C ratio of 1.89.

Relative proportions of transferrin species in Coomassie blue–stained gels and Western blot autoradiograms were evaluated with a laser scanning densitometer (Biomed Instruments, Fullerton, Calif) after the procedures of Chasteen and Williams[16] (Fig 1B). Five scans at uniform spacing across each lane were taken for each sample and the results averaged to minimize errors from a nonuniform distribution of protein across lanes.

For isoelectric focusing the methods given by Huebers and co-workers[15] were followed as closely as possible.

Iron Analyses

Determinations of serum iron concentrations and saturations were performed with Sigma kits (Sigma Chemical Co, St Louis) according to the manufacturer's instructions.

RESULTS

Validation of Present Methods

Efficiency of transfer in Western blots. To evaluate the completeness of protein transfer from gel to nitrocellulose paper during the Western blot procedure, gels were stained with Coomassie blue following electrophoretic transfer. No protein bands could be seen, indicating that transfer from the gels was complete.

Standard curves. A series of eight transferrin solutions varying in saturation from 0% to 100% was prepared. Standard curves relating saturation estimated from densitometry to actual saturation gave a satisfactory linear relationship (Fig 2). Results from Western blot scans agreed with results from Coomassie blue stains of preparations run in parallel but not transferred from gel to nitrocellulose paper. Apotransferrin and saturated transferrin each gave single bands by urea gel electrophoresis, demonstrating that neither contamination of preparations with extraneous iron nor removal of specifically bound iron from transferrin had occurred during the urea gel electrophoresis procedures.

Reproducibility and constancy over time of serum samples. Two samples of different sera, both run in triplicate in each of two separate gels, yielded ratios of N-terminally occupied monoferric transferrin to C-terminally occupied monoferric transferrin (N:C ratio) of 1.63 ± 0.05 and 2.33 ± 0.18. Thus, the reproducibility of the Western blot method appears to be better than ±10%.

Four samples of blood were obtained from one volunteer throughout a period of eight hours. The N:C ratios ranged from 1.06 to 1.22, indicating little diurnal variation in this individual.

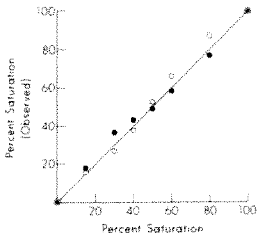

Fig 2. Observed *v* actual transferrin saturations in purified transferrin preparations. ○, Coomassie blue stains; ●, Western blots.

Analysis of Serum Samples

In a series of 21 serum samples from normal volunteers, the ratio of N-terminal monoferric transferrin to C-terminal monoferric transferrin ranged from 0.31 to 6.87 (Fig 3). In 16 subjects this N:C ratio exceeded unity, ranging from 1.06 to 6.87. In five subjects the N:C ratio was less than unity, ranging from 0.31 to 0.79. These subjects tended to have lower levels of serum iron (60 ± 24 μg/dL) than did those subjects with N:C ratios greater than unity in whom the mean serum iron levels were 110 ± 35 μg/dL. Linear regression analysis also indicates a direct, albeit modest, correlation between the serum iron concentration and N:C ratio ($r = .543$).

Studies of Isoelectric Focusing

Preparations of apotransferrin, 50% saturated transferrin, and fully saturated transferrin were examined by isoelectric focusing in polyacrylamide tube gels. None of these preparations gave single or simply interpretable bands (Fig 4). In contrast, urea gel electrophoresis yielded single bands for apotransferrin and diferric transferrin and a four-band pattern for half-saturated protein. One possibility to account for the multiplicity of bands in saturated transferrin is that the protein is partially depleted of iron by the low pH (5.0 to 5.4) at which it electrofocuses. In support of this possibility, we have observed that saturated transferrin in 2% Ampholine at 2 °C loses 25% of its absorbance at 470nm within 15 minutes. This accords with the finding of Surgenor and colleagues, reported nearly 40 years ago,[20] that transferrin relinquishes specifically bound iron below pH 6.3.

DISCUSSION

The N:C ratio measures the relative occupancies of the two sites of transferrin in the circulation, thereby affording a

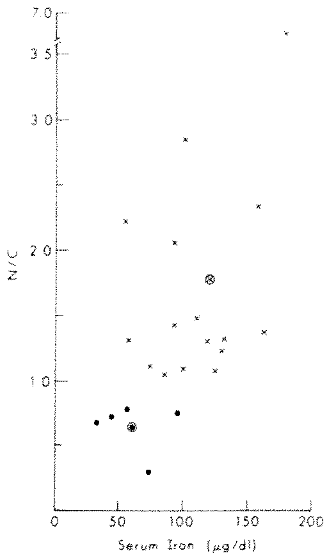

Fig 3. Scatter plot of N:C ratios in 21 normal human serum samples. ●, samples with N:C less than unity; ×, samples with N:C greater than unity. Circled points indicate mean values for each set.

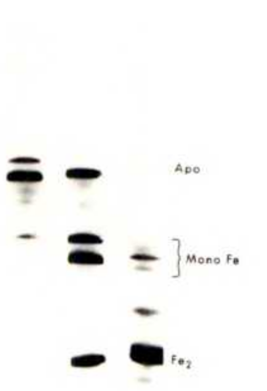

Fig 4. Isoelectric focusing patterns of purified transferrin preparations at indicated saturations with iron. Gels are stained with Coomassie blue.

measure of the apparent affinities of the two sites of the protein for iron[1] in the living organism. For this reason we have expressed our results in terms of this ratio rather than as the equivalent but perhaps less revealing form giving the percentages of total monoferric transferrin in the N and C states. The mean value of the N:C ratio for all subjects tested is 1.60. Accordingly, the binding of iron to circulating transferrin is neither randomly determined, in which case the N:C ratio must be unity, nor thermodynamically governed, for then the N:C ratio must be close to 0.2.[1] Evidently the differential occupancies by iron of the two sites of circulating transferrin reflect physiologic events not yet understood in the loading or unloading of iron by the proteins. Of possible relevance in this context, earlier studies from this laboratory have shown that absorbed iron is preferentially directed to the N-site of transferrin in the intact rabbit.[21]

Our present results are in qualitative accord with those reported earlier from this laboratory[12] and by Williams and Moreton.[13] In our earlier work antedating the introduction of Western blotting, crossed immunoelectrophoresis was used to identify and quantitate transferrin species. This procedure is more difficult to use for multiple samples than the presently employed Western blotting method and its quantitative reliability somewhat uncertain.[22] Furthermore, in the earlier study no precautions were taken to minimize artifacts caused by the improper storage of serum samples,[13] or by urea gel electrophoresis at pH 8.4, which may favor redistribution of iron to the N-terminal site of transferrin.[16] For these reasons, we believe our present findings are quantitatively more reliable than our earlier results in which the N:C ratio in six serum samples ranged from 3.5 to 28. In the study by Williams and Moreton, identification and quantitation of transferrin bands in urea gel preparations run at pH 8.4 and stained with Coomassie blue were made possible by precipitating most other serum proteins with the protein denaturant Rivanol. Using a different method for the estimation of apparent affinities for iron of the two sites of transferrin based on the rate of change of available sites with the change in iron loading rather than the thermodynamic approach we have used, these investigators found an average apparent affinity of the N-terminal site of circulating human transferrin to be 1.38 times greater than that of the C-terminal site, in reasonable agreement with our present result showing a mean N:C ratio of 1.60. The difference between the two values for the relative affinities of N- and C-sites may be due to variations in sample populations, to differences in methods of calculating the ratio, or to differences in methods for resolving and displaying the four forms of transferrin in serum.

Possible explanations for our unsatisfactory results with isoelectric focusing are that apotransferrin may be exposed to extraneous iron in commercial ampholytes that cannot be rendered free of iron and that initially saturated transferrin is partially depleted of iron by the low pH (5.0 to 5.4) at which it electrofocuses. For this reason, studies of transferrin polymorphism by isoelectric focusing, for which the high resolution of the technique is well suited, require that serum samples be overloaded with iron before analysis to insure that all the transferrin is kept in saturated form.[23] Further confounding the interpretation of isoelectric focusing patterns of transferrin is the microheterogeneity of the protein in the circulation resulting from variations in its sialic acid content. This microheterogeneity leads to an overlap of bands from species varying in the number of sialic acid residues with bands from species differing in iron content and distribution.[24] Such difficulties are not encountered in the urea gel electrophoresis technique, which is sensitive to the unfolding of iron-free domains of transferrin[25] rather than to small differences in charge. For these reasons we feel that analysis of the iron distribution in transferrin by urea gel electrophoresis is preferable to analysis by electrofocusing.

A random distribution of iron between the two binding sites of circulating transferrin is one in which each site is equally occupied, and the probability of this occupancy is given by the fractional saturation of transferrin with iron. It

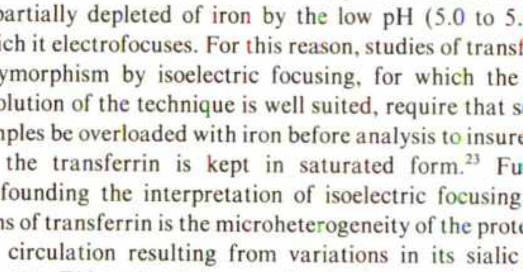

Fig 5. Theoretical plots of transferrin species bearing 0, 1, and 2 atoms of iron per molecule as a function of iron saturation. Calculations are based on the formulations of Chasteen and Williams,[16] assuming no interactions between the two sites of transferrin.

follows, therefore, that in a random distribution the concentrations of N-terminal and C-terminal monoferric transferrins must be equal. More importantly, inferences about random occupancy of the two binding sites of transferrin, founded on graphical representations of data in which only relative proportions of apotransferrin, diferric transferrin, and total monoferric transferrin are offered, as in the report by Huebers et al,[15] are conceptually flawed. From the formulations presented by Chasteen and Williams,[16] it may be shown that random distributions cannot be experimentally distinguished from nonrandom distributions when only total monoferric transferrin is considered, even if the relative occupancy of one site is as much as four times greater than the occupancy of its partner (Fig 5). This is because the relative proportion of total monoferric transferrin changes slowly with a change in the relative occupancies of N- and C-terminal sites, as shown in the series of curves in Fig 5. For iron distribution in circulating transferrin to be truly random, N- and C-terminal sites of the protein must be equally populated, and both monoferric forms must be present in equal concentration. That this is not the case is evident from the wide range of the N:C ratio, from 0.31 to 6.87 in our present study. Clearly, the distribution of iron in circulating transferrin is determined by other than random processes, but the nature of these processes remains unknown.

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Purified Colony-Stimulating Factors Enhance the Survival of Human Neutrophils and Eosinophils In Vitro: A Rapid and Sensitive Microassay for Colony-Stimulating Factors

By C.G. Begley, A.F. Lopez, N.A. Nicola, D.J. Warren, M.A. Vadas, C.J. Sanderson, and D. Metcalf

The survival of purified human blood neutrophils and eosinophils was monitored using microwell cultures. Survival was enhanced in cultures containing human or murine colony-stimulating factors (CSFs). The survival of both cell types was enhanced by purified recombinant human granulocyte-macrophage CSF and partially purified preparations of the native molecule, CSFα. Neutrophil but not eosinophil survival was enhanced by murine granulocyte-CSF and its human analogue CSFβ. Eosinophil but not neutrophil survival was enhanced by murine eosinophil differentiation factor (eosinophil CSF). The mature cell survival provided an assay system for CSF that was 10^2 to 10^3 more sensitive than the standard assay of stimulation of colony formation in agar cultures and could be completed within 48 hours. These results demonstrated that CSF induced and enhanced survival of mature human neutrophils and eosinophils in vitro. Furthermore, the lineage specificity of purified murine CSFs was retained in their action on human cells.

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THE AVAILABILITY of purified native and recombinant colony-stimulating factors (CSFs) has allowed the study of the effects of these molecules on populations of murine and human cells. In keeping with the action of CSFs to stimulate end cell functional activity[1,2] and the survival of progenitor cells,[3-7] evidence has been produced to suggest that CSF may be involved in prolonging survival of mature cells.[8,9]

Two human CSFs with granulocyte-macrophage activity have been identified from several sources of crude conditioned medium based on differences in hydrophobicity.[10-12] In the first eluting activity, CSFα stimulates functional activity of neutrophils and eosinophils whereas CSFβ stimulates neutrophils but not eosinophils.[1,2] Similarly, CSFα stimulates proliferation of neutrophil, macrophage, and eosinophil progenitor cells whereas CSFβ does not stimulate eosinophil colony formation.[6,10] The recombinant human granulocyte-macrophage CSF (rH GM-CSF)[13-15] is thus analogous to CSFα and possesses all the biologic activities previously described for the partially purified native molecule.[16]

An intriguing aspect regarding the action of CSFs is that the lineage-specific murine molecules (granulocyte CSF [G-CSF], eosinophil differentiation factor/eosinophil CSF [EDF/Eo-CSF]) are completely active and retain their lineage specificity on normal human cells.[2,12,17] Similarly, murine macrophage CSF (M-CSF) and human urinary M-CSF display antigenic and biochemical reactivities across species.[18,19] In contrast, neither the rH GM-CSF nor the murine GM-CSF display cross-species reactivities.[2,16,20]

These experiments were performed to examine whether the human-active CSFs could enhance the survival of mature neutrophils and eosinophils and whether this might be used as a rapid and sensitive assay for such CSFs.

MATERIALS AND METHODS

Preparation of purified neutrophils and eosinophils. Peripheral blood was obtained from healthy volunteers with their informed consent according to the guidelines laid down by the Ethics Committee of The Walter and Eliza Hall Institute. Neutrophils and eosinophils were purified on a discontinuous hypertonic metrizamide gradient as previously described.[21] The purity of these preparations was assessed by preparing cytocentrifuge smears and staining with May-Grünwald and 4% Giemsa. In all experiments the cell preparations were >98% pure for neutrophils and >95% pure for eosinophils. In some experiments contaminating red blood cells were removed from the eosinophils by hypotonic lysis in distilled water for 20 seconds. This procedure did not alter eosinophil survival in control experiments.

Microwell cultures of purified neutrophils and eosinophils. All cultures were performed using 60-well Lux 5260 HLA tissue culture plates (Miles Laboratories, Naperville, Ill). Each well contained 10 µL Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 200 cells. Serial dilutions of 5 µL of CSF-containing preparations were added to the microwells prior to the addition of the target cells in the culture medium.

Cultures were incubated at 37 °C in a fully humidified atmosphere of 10% CO_2 in air.

Cultures were examined at intervals and direct cell counts performed on replicate wells using an inverted microscope at 200× magnification. With the optical conditions used (reduced lighting), viable cells had a sharp, clearly defined cell border and were highly refractile, whereas dead cells were dull, had no distinct edge, and often lacked any cytoplasm. Eosin was then added (5 µL) to each well, and cell counts were repeated ten minutes later using eosin exclusion as the measure of cell viability. Results of these two methods were compared.

In control experiments, cell counts were performed by two to four independent observers. Although there was some variation in the absolute cell numbers obtained (between 0% and 20%; mean, 6% for wells containing between 10 and 250 cells), the end points and the slopes of titration curves were the same.

Human marrow cell cultures. Marrow samples were obtained with the prior informed consent of patients undergoing cardiac surgery and according to a protocol approved by the Ethics Commit-

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tee of The Walter and Eliza Hall Institute. Patients had no hematologic disease. Marrow cells were washed in Eisen's balanced salt solution and layered over Ficoll-Hypaque (1.077 g/mL) and centrifuged for 20 minutes at 1,000 g. Interface cells were washed and resuspended in DMEM containing 10% FCS. Cells were cultured in 35-mm Petri dishes using 1 mL of double strength DMEM and a final concentration of 20% FCS and 0.3% agar. A total of 2×10^4 cells were cultured per milliliter. Each culture dish contained 0.1 mL of CSF-containing preparations that were pretitrated to deliver a maximal stimulus and were without high-dose inhibition. Cultures were incubated in a fully humidified atmosphere of 10% CO_2 in air. Colony formation (clones of 40 or more cells) was scored at 35× magnifications using a dissection microscope after seven and 14 days of incubation.

CSF preparations. Human placental conditioned medium (HPCM) and the semipurified CSFα and CSFβ fractions were prepared as previously described[10,22] using phenyl-Sepharose chromatography of medium conditioned by the bladder carcinoma cell line U5637.[12]

The recombinant human GM-CSF (rH GM-CSF), provided by Drs E. Wang, G. Wong, and S. Clark, the Genetics Institute, Boston, was cloned from a cDNA library from the Mo human T leukemia cell line, expressed in monkey CV1 origin SV-40 (COS) cells, and purified as previously described.[13,16,23]

Purified murine G-CSF (6×10^8 U/mg) was prepared from mouse lung conditioned medium as previously described.[24] Murine EDF (Eo-CSF) was obtained from the medium conditioned by a murine T cell hybridoma[25] and purified as described.[26]

All CSF concentrations are expressed as the final concentration of CSF in the culture.

RESULTS

Neutrophil survival in vitro. Figure 1 shows the results of a typical experiment in which the survival of purified peripheral blood neutrophils was examined. For the first 15 hours of the culture period there was no difference between the survival of neutrophils in control cultures compared with cultures containing approximately 10 U/mL of rH GM-CSF, CSFα, or murine G-CSF. In Fig 1, the first time point at which dead cells were noted in control cultures was at 20 hours. The time at which death was first evident varied between 14 and 20 hours for neutrophils obtained from different individuals. On continued incubation, cell numbers continued to decline, and after approximately 35 hours, less than 10% of cells remained in control cultures of neutrophils from eight individuals examined.

Neutrophils in cultures containing CSF showed a consistent enhanced survival compared with cells in control cultures. In the example in Fig 1, this difference was most striking at the 28-hour time point, when the mean number of cells in control cultures was 10 compared with 100 cells in wells containing CSF. There was no apparent difference between rH GM-CSF, CSFα and murine G-CSF in terms of their ability to enhance neutrophil survival.

Eosinophil survival in vitro. The survival of purified human eosinophils in vitro was also enhanced when cells were cultured in the presence of CSF (Fig 2). At early time points there was no difference between control cultures and cultures containing CSF. Dead cells were first observed in control cultures at 26 hours (range, 18 to 28 hours for different individuals). Cell death was progressive in control cultures, and less than 10% of cells remained after approximately 40 hours for all individuals examined. The survival of eosinophils was enhanced in cultures containing CSF. The difference between control and CSF-containing cultures was most evident at the 40-hour time point (Fig 2), when the mean number of cells was 20 in control cultures compared with 108 in CSF-containing cultures. At equivalent concentrations there was no apparent difference between rH GM-CSF, CSFα, and murine EDF (Eo-CSF) in terms of their ability to enhance eosinophil survival in vitro.

Although these results did not necessarily document the maximal in vitro survival time in mature human neutrophils and eosinophils, they did demonstrate that survival in a standardized culture system was enhanced in CSF-stimulated cultures.

Eosinophils consistently showed a longer survival in vitro than neutrophils obtained from the same individual. The mean time taken for 50% of the cells to die in unstimulated cultures was 20 hours (mean of four experiments) for neutrophils and 30 hours for eosinophils.

Viable cell counts were performed on the same microwells in the presence and absence of eosin to test whether cell viability could be accurately assessed by direct visual examination. The results are shown in Fig 3. For neutrophils, cell viability was accurately assessed from the morphology of the

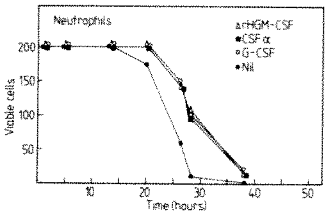

Fig 1. The survival of human blood neutrophils (>98% pure) in vitro in control cultures and cultures containing CSF. Cells were placed in microtiter trays (200 cells per well), and the number of viable cells was counted in four replicate wells at various time points thereafter. Cell survival was enhanced in cultures containing recombinant human GM-CSF (rH GM-CSF), semipurified human CSFα, and murine G-CSF. The concentration of CSF was approximately 10 U/mL as determined by normal human bone marrow colony formation.

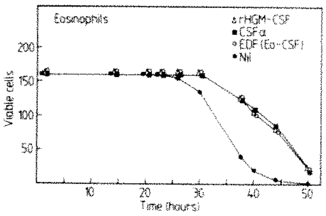

Fig 2. The survival of human blood eosinophils (>95% pure) in vitro in control cultures and cultures containing CSF. Cells were placed in microtiter trays (160 cells per well), and the number of viable cells was counted at varying times thereafter. Results shown are the means of four replicate wells. Cell survival was enhanced in cultures containing rH GM-CSF, semipurified human CSFα, and murine EDF (Eo-CSF). The concentration of CSF was approximately 10 U/mL as determined by normal human bone marrow colony formation.

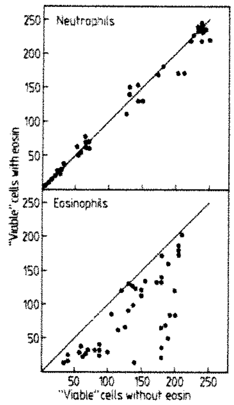

Fig 3. **Cell viability was examined either in cultures without eosin or in cultures with eosin added. Two hundred cells (either neutrophils or eosinophils) were placed in microtiter wells and examined at various time points thereafter. There was no difference in cell survival of neutrophils as assessed by simple inspection or the use of eosin (upper panel). Following the onset of cell death in cultures of eosinophils, eosin was required to accurately assess numbers of remaining viable cells (lower panel).**

cells in the absence of eosin (Fig 3, upper panel). This was because these cells disintegrated at or about the time they ceased to exclude eosin. In contrast, eosinophils maintained their apparent integrity for many hours after they had ceased being able to exclude eosin (Fig 3, lower panel).

Titration of survival-enhancement induced by rH GM-CSF. The activity of varying concentrations of purified rH GM-CSF in enhancing cell survival was examined, and a typical result is shown in Fig 4. Similar results were also obtained for equivalent concentrations of the partially purified native molecule CSFα, which shares all the biologic activities of the rH GM-CSF[16] (data not shown).

The upper panel (Fig 4) shows a titration of the rH GM-CSF at the 28-hour time point when purified neutrophils were used as the target cell population. This molecule was also effective in enhancing eosinophil survival (Fig 4, lower panel), and the responsiveness of the two cell types to CSF was very similar. These results were consistent with previous reports of this molecule being active in stimulating the functional activity of mature cell populations.[1,16] The initial concentration of rH GM-CSF shown in Fig 4 was 670 pg/mL. The lowest concentration of rH GM-CSF detected as enhancing survival was approximately 1.3 pg/mL.

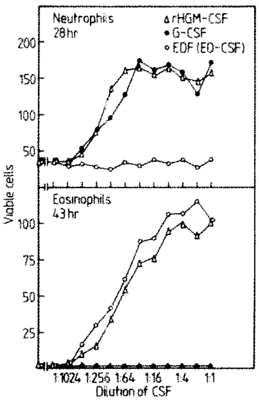

Fig 4. **Dose-response relationship for purified blood neutrophils (upper panel) and eosinophils (lower panel) when cultured with rH GM-CSF, murine G-CSF, and murine EDF (Eo-CSF). Two hundred cells were placed in microtiter trays, and the numbers of viable neutrophils were counted after 28 hours. Viable eosinophils were counted after 43 hours. Results are the means of replicate wells stimulated by twofold dilutions of each CSF preparation. The initial concentration of rH GM-CSF was 670 pg/mL.**

Enhancement of survival of human cells by murine G-CSF and EDF (Eo-CSF). Murine G-CSF and murine EDF (Eo-CSF) are able to stimulate the proliferation of human progenitor cells and functional activity of human neutrophils or eosinophils in a lineage-restricted manner.[2,12,17] The action of these purified murine molecules was examined as shown in Fig 4.

Neutrophil survival was enhanced when cells were cultured in the presence of murine G-CSF. Similar results were obtained when cells were cultured with human CSFβ, the human analogue of murine G-CSF[12] (data not shown). Murine EDF (Eo-CSF) was inactive (Fig 4, upper panel), suggesting that enhanced survival was a specific, CSF-mediated response.

Eosinophil survival was enhanced by murine EDF (Eo-CSF) whereas murine G-CSF and human CSFβ were inactive upon these cells (Fig 4, lower panel). These results confirmed and extended previous descriptions of the activities of these purified murine molecules upon specific populations of human cells and argue strongly against a purely nonspecific survival-enhancing action of the CSFs on these cells.

Morphologic changes induced by CSF. At higher concentrations of CSF both neutrophils and eosinophils underwent a striking morphologic change similar to that previously described for neutrophils, for example, with *N*-formylmethionine-leucine-phenylalanine or influenza virus.[27] Cells ceased to be spherical and assumed irregular shapes, becoming elongated, "tear drop," and convoluted in appearance. These morphologic changes were maximal within one-quarter to one-half hour and reversed over several hours. The viability of these cells subsequently was significantly compromised compared with cells held at lower concentrations of CSF where no such change was observed but where survival was prolonged. The rH GM-CSF and CSFα induced this morphologic change in both neutrophils and eosinophils. Murine G-CSF and human CSFβ induced similar acute morphologic changes in neutrophils but not eosinophils, and the murine EDF (Eo-CSF) induced changes in eosinophils but not neutrophils, indicating that this was a specific result of stimulation by CSF. The concentration of rH GM-CSF above which this morphologic change was observed in >50% of the cells in one hour was approximately 50 pg/mL. Similarly, higher concentrations were required for the other CSFs than the concentrations at which survival was enhanced. Less than 5% of the cells in unstimulated cultures demonstrated this change.

Higher sensitivity of survival assay compared with colony assay. Figure 5 shows the stimulation by purified rH GM-CSF of colony formation by normal human bone marrow cells. This factor preferentially stimulated the day 14

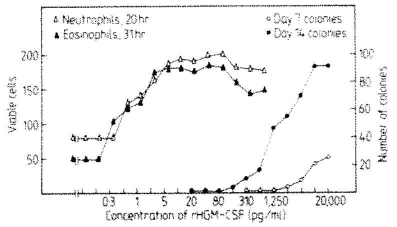

Fig 5. Relative sensitivity of CSF assays during colony formation or survival of mature cells stimulated by rH GM-CSF. A quantity of 2 × 10^4 marrow cells were cultured per milliliter, and colonies (>40 cells) were scored on day 7 and day 14 of culture. The initial concentration of rH GM-CSF was 18,200 pg/mL. The ability of rH GM-CSF to enhance survival of mature neutrophils and eosinophils is also shown. Two hundred cells were cultured in 15-μL volumes and viable cells counted at 20 hours (for neutrophils) and 31 hours (for eosinophils). The initial concentration of rH GM-CSF was 670 pg/mL. All concentrations are expressed as the final rH GM-CSF concentration in the culture.

subset of human colony-forming cells that includes neutrophil, macrophage, and eosinophil colonies.[16] The starting concentration was 18.2 ng/mL of rH GM-CSF. A titration of the rH GM-CSF on neutrophil and eosinophil survival is shown for comparison. The starting concentration was 0.67 ng/mL in the microwell assay. The lowest concentration of rH GM-CSF detected in the colony-forming assay was approximately 280 pg/mL compared with approximately 0.7 pg/mL in the microassay.

The sensitivity of the microassay was greater than that of the colony assay by 5 × 10^2-fold. In other experiments, the greater sensitivity of the microassay ranged between 10^2- to 10^3-fold for the rH GM-CSF. This heightened degree of sensitivity was also seen with CSFα, CSFβ, and murine G-CSF (data not shown).

DISCUSSION

This study has demonstrated that purified CSFs induce enhanced survival of purified populations of mature human neutrophils and eosinophils in vitro. Previous experiments have shown that survival of progenitor cells is dependent upon CSF,[3-7] and these observations have now been extended by the demonstration of a similar type of lineage-specific action for CSF upon mature cells. Furthermore, the lineage specificity of murine G-CSF and murine EDF (Eo-CSF) for murine cells[24,25,28] is retained in their action on human cells. The rH GM-CSF, on the other hand, was shown to stimulate enhanced survival of both neutrophils and eosinophils. The dual lineage activity of this molecule has been previously documented for progenitor and mature cell populations,[16] thus making it analogous to the murine GM-CSF.[29] It is intriguing, however, that despite biologic and sequence homologies neither molecule appears to show a cross-species reactivity.[16,20]

Several microassay systems have been described that are dependent upon proliferation of target cell populations to detect CSF.[20,30-34] These systems are of value because of the small volumes of CSF required and because of the heightened sensitivity of CSF that such murine assays display.[30-33] There is no evidence, however, that proliferation-dependent human microassays are more sensitive to CSF than traditional agar assays.[20,34] The assay described here is thus unique in displaying an increased sensitivity to CSF, between 10^2- and 10^3-fold the sensitivity of agar assays. Furthermore, this assay provides a rapid result but retains lineage specificity for the different CSFs.

The heightened sensitivity to CSF of this microassay compared with other human microassays or agar assays is likely to be the consequence of differences in both the target cell population used and the end point of the assay. Several studies have demonstrated that a heightened sensitivity to CSF is associated with differentiation within progenitor cell compartments since promyelocytes-myelocytes (cluster-forming cells) are more sensitive to CSF than blast cells (colony-forming cells).[35-37] Furthermore, lower concentrations of CSF are required for survival of progenitor cells than the levels required for proliferation.[33] This study has taken advantage of both these observations by using terminally differentiated (and therefore probably the most CSF-responsive) cells and examining cell survival rather than a functional end point.

For progenitor cells, the particular elicited response to CSF (ie, survival *v* proliferation *v* differentiation) is dependent upon the concentration of CSF.[33,38-40] This may also be true for mature cells, since the level of CSF required for the morphologic change of polarity to occur within cells was approximately 10^2-fold the concentration of CSF required for cell survival. This suggests a differential response to varying CSF concentrations by mature neutrophils and eosinophils.

The technique described here provides a rapid, sensitive, specific assay for CSF, and as such, may be useful in examining supernatants obtained from candidate CSF-cDNA clones. The demonstration that mature cell survival is dependent upon CSF has potentially important clinical applications, particularly in areas such as granulocyte transfusion, where viability and function of transfused cells may well be enhanced by the addition of CSF.

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Release of Galactosyltransferase From Human Platelets and a Subset of Monocytes in Culture

By K.E. Hopper, Anita D. Semler, G.V. Chapman, and R.A. Davey

We show that human monocytes and platelets release considerable amounts of galactosyltransferase (GT) in serum-free culture as measured by the amount of incorporation of ^{3}H-galactose into ovalbumin. Enzyme production was the greatest among medium-sized mononuclear cells separated by counter-current elutriation. The cells were adherent and positive for the monocyte-specific monoclonal antibody FMC-32. The activity in the monocyte fractions was not due to platelet contamination as shown from experiments in which platelets or platelet antigens were eliminated. Cell viability decreased by less than 3% during the overnight culture, and results from cell disruption experiments showed that the enzyme was not released from dead or dying cells. Cycloheximide inhibited release during 20 hours culture. Approximately 50% of the enzyme in the cell culture supernatant was pelletable at 105,000 g. Platelets released the enzyme more rapidly than did monocytes and were readily stimulated by thrombin to release more GT. Thrombin also increased monocyte GT activity after overnight incubation, but other stimulants, zymosan and lipopolysaccharide (LPS), decreased release. We conclude that GT is released into culture supernatants by platelets and by a subset of peripheral blood monocytes. These sources may account for a significant proportion of the serum enzyme and may be important in modification of extracellular carbohydrates during inflammation and coagulation.

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MANY CELLULAR interactions and functions depend on extracellular carbohydrates, either as cell surface components or as parts of secreted glycoproteins.[1] Enzymatic or chemical modification of the carbohydrate leads to changes in these reactions such as lymphocyte blastogenesis induced by periodate[2] or neuraminidase and galactose oxidase[3] or clearance of desialated glycoproteins by macrophages or hepatocytes.[4] Ectogalactosyltransferases are normal components of serum[5] and are elevated in patients with cancer,[6] liver damage, and acute inflammation.[7] They are secreted by tumor cells[8] and are postulated to be markers for the presence of cancer.[5,9] Hepatocytes[10] and macrophages[11-13] have also been suggested as normal sources of extracellular galactosyltransferase (GT).

We have shown in mice that peritoneal macrophages induced in the early phases of inflammation after a single injection of thioglycollate broth or salmonella release large amounts of GT in culture.[13] Inflammation induced by secondary infection or immune responses in the peritoneal cavity did not lead to release of enzyme. Our studies suggested that the secreting cells in mice were platelets and newly recruited monocytes. We show here that platelets and a subpopulation of small monocytes are responsible for most of the enzyme released by normal human peripheral blood cells in culture.

MATERIALS AND METHODS

Preparation of cells. Citrated human peripheral blood was diluted in the ratio of 1:2 with Hanks' balanced salt solution without Ca^{++} or Mg^{++} (HBSS, GIBCO, Grand Island, NY) in 50 mL polypropylene centrifuge tubes (Crown Corning, Chicago). This was underlayed with Lympho-paque (Nyegaard & Co, Oslo) in a ratio of one part to three parts diluted blood and centrifuged for 40 minutes at 300 g and 18 °C. The buffy interface was recovered, diluted fivefold with HBSS without Ca^{++} or Mg^{++}, centrifuged, and the cell pellet resuspended and washed twice. Neutophils of $\geq$99% purity were prepared as described previously.[14] Citrated blood was centrifuged at 200 g for ten minutes. The platelet-rich plasma was removed and spun at 800 g for 20 minutes, the plasma discarded, and the pellet dispersed and washed (three times) with acid-citrate-dextrose. The platelets were finally washed in HBSS without Ca^{++} or Mg^{++}.

Cell counting. Cell viability and number were estimated by Trypan blue exclusion. Slide preparations (Cytospin, Shandon Southern Products Ltd, Runcon, England) were stained with May-Grünwald Giemsa or for nonspecific esterase.[15]

Separation of cells by counter-current elutriation (CCE). Peripheral blood mononuclear cells were separated by CCE using a Beckman 6.0 rotor with a standard chamber in a J-6B centrifuge (Beckman Instruments, Inc, Palo Alto, Calif) and fitted with a Masterflex peristaltic pump and silicone tubing (Silastic, Dow Corning Corp, Midland, Mich). The rotor chamber was treated with silicone solution (Desicote, Beckman) before rotor assembly, and then 70% ethanol (400 mL), saline (1,000 mL), and elutriation medium (200 mL) were pumped through the apparatus with the rotor running at 2,200 rpm. The elutriation medium consisted of HBSS without Ca^{++} or Mg^{++} containing 0.4% bovine serum albumin (Cohn Fraction V, CSL, Parkville, Australia) and 0.38% sodium citrate. The pump was calibrated immediately before introduction of the sample and pump speeds adjusted to give appropriate corrected flow rates for each fraction. The cell sample (50 to 200 × 10^6 mononuclear cells in 10 mL HBSS without Ca^{++} or Mg^{++}) was applied via a 30-mL loading chamber at a pump speed of 8.0 mL/min. Samples of 100 mL were collected into cold polypropylene tubes from the start of sample application and each filled tube stored on ice. The pump speed was increased stepwise from 8.0 to 22.0 mL/min at a constant rotor speed of 2,200 rpm and then the rotor stopped to elute the remaining cells. After the run, the assembly was washed through with 5% detergent (200 mL, Solution 555, Beckman) followed by 2 L water and left to dry.

The fractions were centrifuged for ten minutes at 300 g and 4 °C, washed twice with cold HBSS without Ca^{++} or Mg^{++}, and then made up to 2 mL with Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 100 µg/mL streptomycin and 100

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U/mL penicillin. Total cell recovery was 70% to 90% of the starting population.

Cell separation in EDTA-containing media. In experiments designed to totally eliminate platelets from the monocytes, the cell preparation (after the Lympho-paque separation) and the elutriation were carried out at 4 °C in media containing 0.5 mmol/L EDTA rather than citrate. The pump speeds were adjusted to give fractions of similar composition to those in media containing citrate. All other experiments were carried out using cells prepared in citrated medium.

Adherence of cells. The cells (3 × 10^5) in 100 μL DMEM containing 10% heat-inactivated human AB serum (56 °C for 30 minutes) were incubated for 90 minutes at 37 °C in 96-well flat-bottomed Linbro microtiter plates (Linbro Scientific Co, Inc, New Haven, Conn). The wells were gently washed with warm DMEM and the nonadherent cells aspirated. The washings from three aspirations were pooled, centrifuged, and 100 μL of DMEM was added to the nonadherent cells and adherent cells.

Cell culture. Mononuclear cells and neutrophils were incubated in Linbro flat-bottomed 96-well plates at 3 × 10^5 cells per well in 100 μL DMEM without serum supplement. Platelets were cultured at 10^7 cells per well in DMEM unless otherwise specified. In some experiments cultures contained human thrombin (Sigma Chemical Co, St Louis), lipopolysaccharide (type W from *Escherichia coli*, Difco Laboratories, Detroit Mich), or zymosan (Zymosan A from *Saccharomyces cerevisiae* yeast, Sigma) prepared in DMEM.

GT assay. GT was assayed as described previously[13,16] using 2.4 μmol uridine diphosphate-[^{3}H]-galactose (The Radiochemical Centre, Amersham, England) as the substrate and 1.8 mg ovalbumin as the acceptor in a total volume of 0.15 mL. Samples of normal serum assayed under the same conditions as culture supernatants gave values of GT activity of 20,000 to 40,000 cpm per 6 μL.

Disruption of cells. After specified incubation periods, (1) Triton X-100 (Sigma, St Louis) (0.5% final concentration) was added to the cells and incubated for 30 minutes at 37 °C, or (2) the cells in 1.5-mL polypropylene centrifuge tubes (Mallinckrodt Ltd, Clayton, Australia) were frozen in liquid nitrogen and thawed at room temperature (3 times) before assay. The cells were totally disrupted after Triton treatment and killed but partially intact after freezing and thawing.

Use of monoclonal antibodies. The monocyte-specific monoclonal antibody FMC-32[17] and platelet-specific monoclonal HuPl-M1[18] were obtained from Australian Monoclonal Development (Artarmon, Australia). The antiplatelet monoclonal PLT-1 was purchased from Coulter Electronics, Inc, (Hialeah, Fla). The cells (1 to 3 × 10^6) were prepared in phosphate-buffered saline containing 1% heat-inactivated human AB serum (PBS + serum). The pelleted cells were incubated with 50 μL of a 1:200 dilution of the antibodies for 30 minutes on ice, washed three times with 1 mL PBS + serum and incubated with 50 μL of a 1:50 dilution of fluorescein isothiocyanate (FITC) conjugated, F(ab′)$_2$ fraction of sheep antimouse Ig (Silenus Laboratories Pty, Ltd, Dandenong, Australia). The cells were washed three times in cold PBS + serum. Control cultures contained either no first antibody or normal mouse immunoglobulins (Coulter Electronics). Wet slide preparations of the cells were viewed at 400× magnification.

Platelets were also identified in separate cytospin slide preparations after staining with monoclonal antibody to factor VIII–related antigen and peroxidase-conjugated goat antimouse Ig (Dakoplatts, Glostrup, Denmark).

Flow cytometry. Analysis and sorting were performed on a Coulter EPICS V flow cytometer (Coulter Electronics) equipped with a Spectra physics Argonion laser (Mountainview, Calif) operated at 488 nm and a power of 400 mW. Two-parameter histograms of forward-angle light scatter (FALS) *v* log-integrated green fluorescence (LIGFL) were collected gated on FALS to exclude cell debris and clumps. Routinely, data on 10^4 cells were collected and analyzed.

RESULTS

Identification of cells secreting GT. Peripheral blood mononuclear cells were separated by CCE, and pooled fractions were cultured overnight in serum-free conditions. As shown in Fig 1, high activity was detected in the supernatant of cells in fraction 1 and among the medium-sized cells in fractions 4, 5, and 6. Cell viability remained greater than 95% after incubation for all fractions except fraction 1, which was 91%.

Fractions 4, 5, and 6 consisted of a mixture of large lymphocytes and small monocytes, and the enzyme release increased with cell number in culture (Fig 2A). Low levels of enzyme were produced by small lymphocytes in fractions 2 and 3 and decreasing amounts by cells in fractions 7 and 8, which were enriched for large monocytes. Fraction 8 also contained some polymorphonuclear leukocytes. Neutrophils prepared by density centrifugation over metrizamide did not release large amounts of enzyme (Fig 2A).

The predominant cells in fraction 1 were platelets. Similar amounts of enzyme were released from this fraction as from the same number of platelets prepared from platelet-rich plasma and purified by repeated low-speed centrifugation (Fig 2b).

Identification and separation of monocytes. To determine whether large lymphocytes or small monocytes in the middle fractions of the elutriation were responsible for

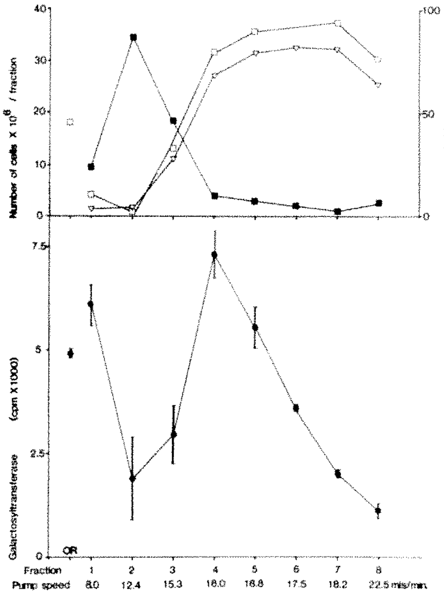

Fig 1. Separation of peripheral blood mononuclear cells by elutriation and release of GT in culture. Figure 1A shows the number of viable nucleated cells per fraction (-■-) and the percentage of monocytes in each fraction identified by morphology (-□-) or esterase staining (-▽-). Figure 1B shows the amount of GT released in culture by 3 × 105 cells from each fraction. Data points represent the mean ± SD of values from triplicate cultures. OR is the unseparated population.

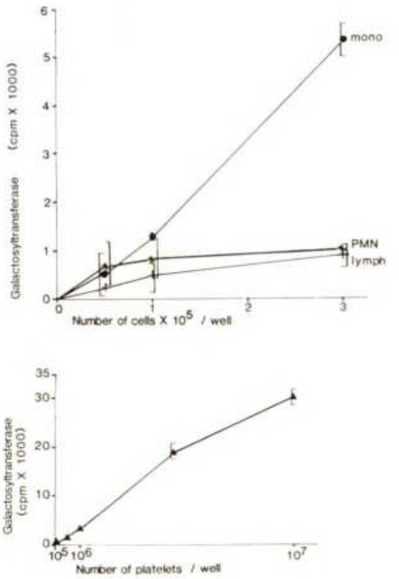

Fig 2. Release of GT from enriched cell populations. Figure 2A shows the GT released from monocytes prepared from elutriation fractions 5 and 6 (-●-), lymphocytes from fraction 2 and 3 (-+-), and neutrophils from metrizamide separation of dextran-sedimented blood cells (-#-). Figure 2B shows the GT released from platelets isolated from platelet-rich plasma.

releasing GT, the cells were separated further by flow cytometry or adherence. Monocytes were separated from other cells in fractions 5 and 6 by flow cytometry using the monocyte-specific monoclonal antibody FMC-32. The separated FMC-32–positive cells, comprising 80.0% of the cells in the fraction, were enriched for monocytes (>98% based on morphology). Most enzyme was released from the FMC-32–positive cells during overnight culture, whereas the unseparated cells released low amounts of GT (Fig 3).

Cells from all fractions were allowed to adhere to plastic for 90 minutes in the presence of heat-inactivated AB serum. The nonadherent cells were removed and the adherent, nonadherent, and unseparated populations washed and cultured for 16 hours to determine the release of GT. Adherence was effective in separating those cells releasing GT from inactive cells in fractions 5 to 8 (data for fraction 5: adherent cells, 2,603 ± 303 cpm; nonadherent cells, 283 ± 207 cpm; and unseparated cells, 4,166 ± 62 cpm). The amount of enzyme recovered from both adherent and nonadherent cells did not total that released by the unseparated population, suggesting that the adherence step affected the release of enzyme or that some active cells were lost.

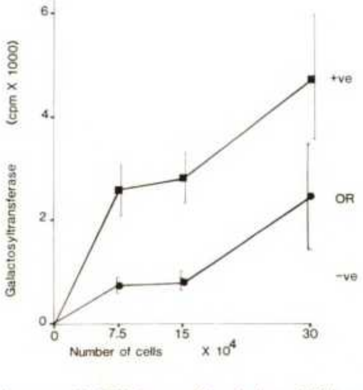

Fig 3. Release of GT by cells during 18-hour culture of FMC-32–positive sorted cells (-■-) and the unseparated populations (-●-).

The adherence treatment was found to be generally less effective in isolating monocytes from fractions 4 and 5 compared with fractions 6, 7, and 8. Loss of monocytes by binding to vessel walls was also a constant problem in fractions 7 and 8 but not in fractions 5 and 6, suggesting that monocyte adherence is greater among cells eluting later in the separation.

Identification and separation of platelets. Decreasing numbers of platelets were observed and clearly distinguished from other cells in fractions 1, 2, and 3, and few were observed in fractions 4 to 8. Using the platelet-specific monoclonal antibodies HuP1-M1 and PLT-1, no free, positive platelets were detected in fractions 4 to 8 by flow cytometric or microscopic examination. The majority of monocytes, however, were weakly positive for this antigen. Cytospin preparations of cells stained with factor VIII–related antigen did show some intact platelets bound to about 10% of the monocytes. These results suggested that the flow cytometric analyses had detected platelet antigens or fragments rather than intact platelets on the monocyte surface.

To determine whether the GT from monocyte fractions had come from monocytes or platelets, the cell preparation and elutriation conditions were changed (using EDTA and low temperature instead of citrate at 18 °C) to give a more stringent prevention of monocyte-platelet adhesion (see Materials and Methods). The monocyte-enriched fractions were totally free of platelet antigens as determined by flow cytometry using PLT-1 (Fig 4) and HuP1-M1 (data not shown) and microscopy using factor VIII–related antigen. GT production in culture was again greatest from monocyte-enriched fractions, and similar total amounts of GT were released from cells prepared in citrate or EDTA. However, using EDTA, there was no difference in GT release per monocyte by cells across the elutriation profile. When 0.5 mmol/L EDTA was included in the culture of cells prepared

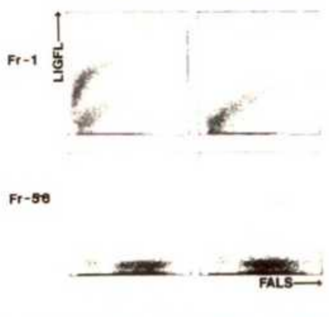

Fig 4. Two-parameter flow cytometry histograms of cells in fraction 1 and pooled fraction 5 to 8 obtained by elutriation at low temperature in EDTA-containing media. Flow cytometry patterns show light scatter on the abscissa and log fluorescent staining on the ordinate. The cells were stained indirectly with PLT-1 or second antibody alone.

in citrated elutriation medium, there was an increase in the release of GT (3,125 ± 234 cpm with EDTA and 2,046 ± 761 without EDTA).

Effect of cell disruption on release of GT. In agreement with previous experiments using mouse peritoneal macrophages, a significant proportion of the released enzyme from human monocytes was pelletable at 105,000 g (3,083 ± 636 cpm in the supernatant before and 2,087 ± 280 cpm after centrifugation). Enzyme activity in culture supernatants from platelets was also decreased by 25% by centrifugation. Some of the enzyme may therefore be released in membrane fragments from dying cells. Maintenance of high cell viability in culture would argue against this. However, to determine the potential of the cells to release preformed enzyme, cells in fractions 5 and 6 were cultured for four or 20 hours and then treated with Triton X-100 or were frozen and thawed (three cycles) (Fig 5). The lysates were centrifuged at 200g and the GT in the supernatants assayed. Consistently more enzyme was released after treatment with Triton, and there was little increase in GT with time of culture. The relatively low amount of enzyme available after Triton treatment suggested, however, that the enzyme released in culture was not coming from dead cells.

Freezing and thawing did not release large amounts of enzyme. There was no difference in the amount of released GT if the frozen-thawed cells were centrifuged at 200 g, and very little enzyme was detected if the cells were centrifuged before lysis.

Cycloheximide at 10^{-5} mol/L included in the cell culture partially inhibited the release of GT overnight (1,410 ± 259 cpm with, 2,916 ± 52 without inhibitor), but not during the four-hour culture (1,447 ± 323 with, 725 ± 65 without inhibitor). Lower concentrations of inhibitor had no effect at either time.

Effect of cell stimulation. Platelets prepared from fraction 1 and mononuclear cells from pooled fractions 5 and 6 were incubated with thrombin for two hours or 16 hours. As shown in Fig 6, thrombin caused a marked increase in GT release from platelets but not monocytes during the two-hour incubation. When monocytes were incubated for 16 hours with thrombin, there was a significant increase in release. In other experiments the effect of thrombin on platelets was detected within minutes of addition (data not shown), and results for 4, 6, or 16 hours were similar, indicating that for a given amount of thrombin the platelets were probably maximally stimulated very early during the incubation. In control experiments, thrombin was added to preformed supernatants from cultures of monocytes or platelets to determine the effect of the protease on GT. There was a 30% reduction in the activity of the monocyte supernatant and a 10% reduction in the activity of the supernatant from platelets cultured more than 16 hours.

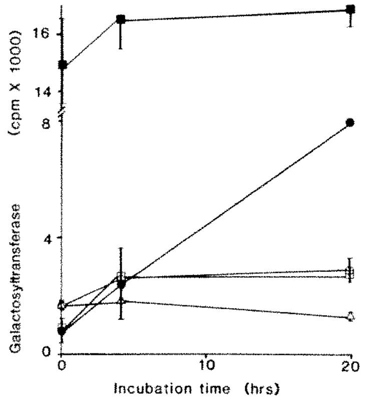

Fig 5. Effect of cell disruption on release of GT. Cells in elutriation fractions 5 and 6 were cultured and treated with Triton X-100 (-■-) or frozen and thawed (open symbols) and the amounts of GT released compared with that from untreated cells in culture (-●-). Three protocols of freezing and thawing are shown: (-○-), the cells were cultured, frozen, and thawed and the whole lysate assayed; (-□-), the lysate was centrifuged at 800 g and the supernatant assayed; (-△-), after incubation the cells were washed twice and then lysed.

Opsonized zymosan and lipopolysaccharide (LPS) are potent monocyte stimulants, and in other experiments treatment of monocytes with LPS increased tumoricidal and procoagulant activities, whereas opsonized zymosan stimulated the generation of chemiluminescence (Andrews and Hopper, data not shown). As shown in Fig 7, however, LPS caused a reduction in GT release from monocytes and, to a lesser extent, from platelets cultured more than 18 hours. Similar results were obtained using opsonized zymosan or both agents incubated for four hours.

DISCUSSION

The liver is believed to be the major source of GT in blood.[7] We conclude from this study that normal human platelets and a subset of small monocytes from peripheral blood release considerable amounts of GT into the supernatant of serum-free cultures. If similar GT production occurs in vivo, this would account for a small but significant proportion (probably about 5% based on cell numbers) of the free enzyme in normal blood. These results confirm, and extend

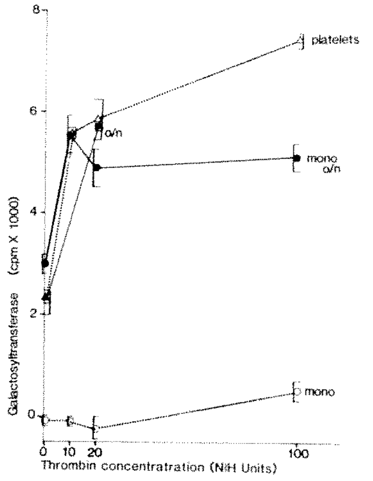

Fig 6. Effect of the addition of thrombin to platelets (triangles) (fraction 1, 10^7) and monocytes (circles) (fraction 5 and 6, on release of GT during culture for two hours (△▲) or 16 hours (○●).

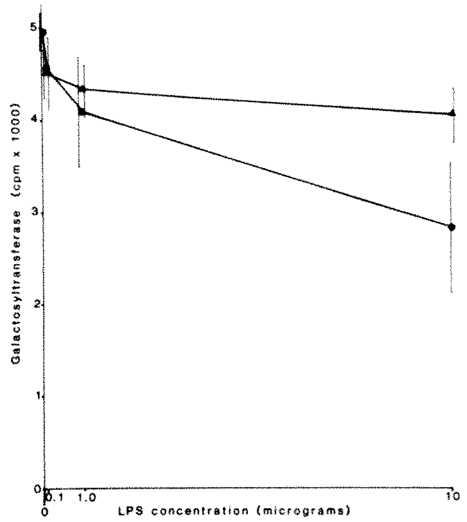

Fig 7. Effect of LPS on release of GT from platelets (▲) and monocytes (●) during an 18-hour incubation.

to humans, our previous studies using mice in which we showed that subpopulations of monocytes/macrophages that had been recently recruited to an acute inflammatory reaction in the peripheral cavity produced large amounts of this enzyme.[13] Conditions for the release of GT appear to be the same in both species. We postulated in the case of the mouse that the secreting cells may constitute a subpopulation of monocytes or be in a particular activation state that is induced in acute inflammation. This may lead to local concentration of the enzymes.

Monocyte subpopulations have been separated in several[19,20] but not all previous studies[21,22] using CCE. We confirm that CCE can separate monocyte subsets that have different adherence and ability to release GT. It should be noted that, using low temperature and EDTA in the elutriation, all monocytes were equally capable of GT release and therefore differ from the cells prepared in citrate. This may be due to an effect of these conditions on enzyme release or on different resolution of cell subsets.

Loss of monocytes by adherence to vessels and tubes is a major problem in these separations, and from our observations, this is more serious for monocytes of high sedimentation velocity. In an attempt to overcome these problems, the cells were prepared and collected as quickly as possible using new polypropylene tubes with citrate or EDTA in the Ca^{++}- and Mg^{++}-free elutriation media and after separation keeping the cells at 0 to 4 °C. Citrate was the preferred chelator in the elutriation media because of the tendency of EDTA to cause vacuolation at higher concentrations.[23]

Monocytes prepared conventionally over discontinuous gradients are known to be contaminated by platelets, either as aggregates or bound to monocytes.[24] We have found, in addition, that contamination by platelet fragments is more extensive than by intact platelets and that almost all monocytes, but not other cells, have platelet antigens on their surface. Platelets could therefore be the source of the released GT in the fractions separated by CCE. Several results, however, would argue against this. (1) In confirmatory experiments platelet aggregation and contamination of fractions 4 to 8 were essentially eliminated by strict Ca^{++}- and Mg^{++}-free conditions and low temperature, whereas GT production was not diminished in these monocyte-enriched fractions. (2) Enzyme was released more rapidly from platelets, and these cells were much more sensitive to the presence of thrombin than monocytes during the two-hour incubation. (3) Serum GT exists as a family of enzymes that are separable by isoelectric focusing.[9] We have found that cell culture supernatant GT shows qualitatively similar focusing patterns to serum, but with major differences in amounts of some isoenzymes (Davey and Hopper, manuscript in preparation). The focusing patterns of GT released from platelets differed from that released by monocytes.

Since GTs are membrane-bound intracellular enzymes, it is possible that the released enzyme may come from dead cells that had disintegrated. This does not appear to be the case because cell viability remained high throughout the incubation (above 95%) and complete solubilization of the cells with Triton X-100 only doubled the amount of available enzyme rather than an expected severalfold increase if all cells were equally capable of producing GT. There was, however, a net loss of cellular enzyme during incubation.

Freezing and thawing did not release more enzyme than that obtained from the viable cell culture. There was a decrease in the supernatant enzyme, however, if the cells were cultured and then lysed. This result is probably due to incomplete disruption of the cell membranes and lack of solubilization of membrane-bound enzyme during the freezing and thawing compared with the lysis with detergent. In comparison, mouse cells did release more GT after freezing and thawing and the results suggested the presence of a pool of free enzyme.[13]

The rapid release of GT from platelets and their sensitivity to the effect of thrombin suggest that the enzyme arises by degranulation of these cells. The mechanism of release of GT from monocytes is not understood. Some of the GT released from platelets and monocytes was pelletable at 105,000 g and therefore may be liberated within vesicles during membrane turnover. Adherence, phagocytosis of opsonized zymosan particles, and incubation with LPS reduced the amount of enzyme released, and these processes would be expected to alter membrane stability and turnover. Increased membrane fluidity may be a property of the monocyte subset responsible for GT release. Under the same conditions as used here, monocytes showed procoagulant activity when stimulated by LPS and chemiluminescence by opsonized zymosan (Andrews and Hopper, unpublished observations). Typical techniques for the stimulation of monocytes, therefore, did not promote release of GT. Thrombin did, however, cause an increased release of GT from monocytes during overnight culture. Thrombin is also known to bind to monocytes[25] and act as a chemoattractant.[26] An interesting hypothesis for the mechanism of release of GT may be the conversion of membrane-bound enzyme to an active hydrophilic form by limited proteolysis.[27] Such a mechanism would also be in agreement with our previous data on the effects of thrombin and trypsin on mouse cells and the lack of effect of other metabolic inhibitors. Cycloheximide was only partially effective in inhibiting release after 20 hours and was not inhibitory after four hours, suggesting that the enzyme may not be immediately available for release after synthesis.

Platelets and monocytes are involved in many related aspects of inflammatory and coagulation reactions. Since in this paper we show that significant amounts of GT are released from both these cell types, it may indicate that GT is an additional mechanism for modification of extracellular carbohydrate.

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Analysis of Human Platelet Glycoproteins IIb-IIIa and Glanzmann's Thrombasthenia in Whole Blood by Flow Cytometry

By Lisa K. Jennings, Richard A. Ashmun, Winfred C. Wang, and Michael E. Dockter

Antibodies that bind to human platelet membrane glycoproteins IIb and IIIa were used to develop methods for analyzing platelet membrane components by flow cytometry. Platelets were tentatively identified by their low-intensity light scatter profiles in whole blood or platelet-rich plasma preparations. Identification of this cell population as platelets was verified by using platelet-specific antibodies and fluorescein-conjugated antiimmunoglobulin. Two-parameter analysis of light scatter versus fluorescence intensity identified >98% of the cells in the "platelet" light scatter profile as platelets due to their acquired fluorescence. Both platelet-rich plasma and whole blood were used to study platelet membrane glycoproteins IIb and IIIa on a single cell basis in an unwashed system. Prostacycline was included in these preparations as a precautionary step to inhibit platelet aggregation during analysis. Flow cytometry is a successful technique for rapid detection of platelet membrane defects such as Glanzmann's thrombasthenia. Platelets from Glanzmann's thrombasthenic individuals were readily distinguished from platelets with normal levels of glycoprotein IIb and IIIa and from platelets with glycoprotein levels characteristic of hetrozygote carriers of this disorder. This technique provides a sensitive tool for investigating platelet functional defects due to altered expression or deficiency of platelet surface proteins.

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FLOW cytometry is a technique that is being increasingly used to analyze individual components in a homogeneous or heterogeneous population of cells. Analysis by flow cytometry has been used successfully to quantitate cellular DNA and ploidy frequency distributions of cells, particularly those residing in the bone marrow, and has been increasingly used for analysis of ligand-receptor interactions (for review, see references 1 and 2). Flow cytometry may provide one of the best overall methods for analysis of receptor proteins as this technique permits rapid and sensitive analysis of cell surface proteins on a single cell basis.

We have developed a method for analyzing platelet surface components in platelet-rich plasma and whole blood using flow cytometry. Platelet membrane glycoproteins IIb and IIIa were detected on the platelet surface by using as probes monoclonal or heterologous antibodies specific for these glycoproteins. The level of glycoproteins IIb and IIIa on platelets from normal donors was compared to that of platelets obtained from Glanzmann's thrombasthenic individuals. Platelets from individuals with Glanzmann's thrombasthenia and heterozygote carriers for this disorder were easily distinguished by their relative fluorescence intensity from platelets of normal donors. During our studies, we also observed that all cells within the platelet population displayed decreased amounts of glycoproteins IIb and IIIa (characteristic of this inherited platelet disorder), thus confirming that this is a homogeneous defect. Platelet analysis by flow cytometry provides an alternative rapid diagnostic procedure for Glanzmann's thrombasthenia as well as for the detection of other functional defects caused by an altered expression of platelet surface components.

MATERIALS AND METHODS

Chemicals and Materials

Acrylamide, n,n'-methylene bis-acrylamide, ammonium persulfate, bromophenol blue, sodium dodecyl sulfate (SDS), Coomassie brilliant blue, and molecular weight standards were obtained from Bio-Rad (Richmond, Calif). Lactoperoxidase was a generous gift from Dr M. Morrison (St. Jude Children's Research Hospital, Memphis, Tenn). Na^{125}I was purchased from New England Nuclear (Boston, Mass). All other chemicals were purchased from Sigma Chemical Co (St. Louis, Mo) and were of reagent grade. RP Trimax x-ray film was purchased from Eastman Kodak Company (Rochester, NY).

Platelet Donors

RG, a 17-year-old male, had bruised extremities at birth and a subsequent history of gingival bleeding. Glanzmann's thrombasthenia was diagnosed at age 1½ years. Laboratory evaluation showed bleeding time >15 minutes; clot retraction 21%; a platelet count of 193×10^9/L; and platelet aggregation absent with ADP, epinephrine, thrombin, and collagen. Parent SJ has no history of a bleeding disorder.

BL, a 5-year-old male, has had easy bruising since age 9 months. Glanzmann's thrombasthenia was diagnosed at age 2 years. Laboratory evaluation showed bleeding time 9.5 minutes; a platelet count of 280×10^9/L; and no aggregation to ADP, collagen, or epinephrine. He is the offspring of a consanguineous sibling relationship. Parent DL has no history of a bleeding disorder.

Patient EA, a 34-year-old female, has been described in an earlier publication.[3] Laboratory evaluations have shown a prolonged bleeding time; normal platelet count and morphology; impaired clot retraction; and aggregation absent with ADP, thrombin, collagen, or epinephrine.

Antiplatelet Antibodies

An antihuman platelet membrane glycoprotein IIbβ monoclonal antibody (GTB18) was used as a specific probe for human platelets. The preparation and characterization of this monoclonal antibody was described previously.[4] Heterologous Protein A affinity-purified rabbit antihuman platelet glycoprotein IIb-IIIa antibody was kindly

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provided by Dr David R. Phillips, Gladstone Foundation Laboratories (San Francisco, Calif). This antibody was demonstrated to be specific for glycoproteins IIb and IIIa by immunoprecipitation of solubilized platelet membranes and by the Western blot procedure using solubilized membranes or solubilized washed platelets electroblotted onto nitrocellulose paper.[5]

Fluorescein-conjugated (FITC) goat antirabbit and rabbit antimouse immunoglobulin were obtained from Sigma Chemical Co and had fluorescein-to-protein ratios of 4.7 and 4.2, respectively.

Preparation of Platelets

Venous blood from normal adult donors was collected by venipuncture through a 19-gauge needle and mixed with 0.16 volume of a solution containing 0.085 mol/L sodium citrate, 0.111 mol/L dextrose, and 0.071 mol/L citric acid (ACD). Donors took no medication for 2 weeks prior to blood collection.

The blood was centrifuged at 160 × g for 20 minutes, and the platelet-rich plasma removed. The platelets were separated from the plasma and washed twice in CGS buffer containing 0.12 mol/L sodium chloride, 0.013 mol/L trisodium citrate, and 0.03 mol/L dextrose, pH 7.0.[6] The platelets were washed a final time in TS buffer containing 0.154 mol/L sodium chloride, 0.01 mol/L Tris, and 0.0001 mol/L leupeptin, pH 7.4. The platelets were suspended in TS buffer for surface labeling. All procedures were conducted at room temperature.

Iodination of Platelet Surface Proteins

Platelets were radiolabeled with ^{125}I by lactoperoxidase-catalyzed iodination using a previously published procedure.[7] Washed platelets were solubilized in 2% SDS (w/v) containing 2% 2-mercaptoethanol (v/v) incubated at 100 °C for 10 minutes to assure solubilization. Protein (10 to 50 μg) was electrophoresed through slab gels according to the method of Laemmli[8] by using a 5% to 20% expontential gradient of acrylamide in the resolving gel and 3% acrylamide in the stacking gel. Protein was stained by Coomassie brilliant blue. For determination of the distribution of radioactivity, the gels of radiolabeled platelets were dried under vacuum on filter paper and stored next to RP Trimax x-ray film. After 24 to 72 hours exposure, the film was processed according to manufacturer's instructions. The relative amount of radioactivity in glycoproteins IIb and IIIa was determined by integration of the peaks corresponding to the glycoproteins from scans of autoradiographs using a GS 300 Transmittance/Reflectance scanning densitometer interfaced with an Apple IIc computer for analysis (Hoefer Scientific Instruments, San Francisco, Calif).

Preparation of Samples for Flow Cytometry

Platelet-rich plasma preparation. Platelet-rich plasma (PRP) from freshly drawn blood was collected by low-speed centrifugation at 800 × g and then diluted 1:1 (v/v) with CaTS buffer (0.154 mol/L sodium chloride, 0.01 mol/L Tris, and 0.0005 mol/L calcium chloride, pH 7.4) containing 50 μmol/L prostacyclin (PGI_2). To 200 μl PRP-CaTS, an antihuman platelet membrane glycoprotein monoclonal antibody (2 μg) was added and incubated for 30 minutes at room temperature (22 °C). The addition of two micrograms of antibody was shown to be saturating for the number of platelets in the sample. FITC-conjugated rabbit antimouse IgG (10 μg) was added and incubated for 10 minutes.

Preparation of whole human blood. Fresh human blood was diluted 1:1 (v/v) with CaTS buffer containing 50 μM PGI_2. Within 1 hour of venipuncture, the antiglycoprotein IIb and IIIa antibody (10 μg) was added to 400 μL of blood-CaTS and incubated for 30 minutes at room temperature. FITC-goat antirabbit IgG (40 μg) was added and after 10 minutes, the cells were analyzed by flow cytofluorimetry. Control experiments for both PRP and whole blood studies were treated in an identical manner except that buffer or a species-specific IgG (2 μg) was added instead of the antiplatelet antibody.

Flow cytometry. A Coulter EPICS V flow cytometer (Coulter Electronics, Inc) equipped with a Spectra Physics 164-05 argon ion laser adjusted to deliver 800 milliwatts at 488 nm was used to analyze platelets. Cells in suspension were analyzed at a flow rate of approximately 1,000 cells/sec. Forward-angle light scatter (1° to 19°) was converted from linear to logarithmic scale by using a log amplifier of the instrument. Fluorescein fluorescence emission was detected after passage through a 510 nm long pass interference filter to exclude scattered laser light. The light scatter and green fluorescence signals from each cell were collected from 50,000 cells and stored and analyzed by the Coulter MDADS minicomputer analysis system as two-parameter 64 × 64 channel histograms. Routinely, the fluorescence signals measured were gated on a light-scattering profile that excluded contributions from debris. Data (log light scatter *v* log green fluorescence signal *v* cell number) were transferred to a Data General MV8000 computer and accompanying Techtronics 4662 plotter for subsequent graphics.

RESULTS

Antibody binding to platelet membrane glycoproteins was assessed by fluorescence microscopy. Using standard fluorescein filter combinations for fluorescence microscopy, platelets plus antiplatelet glycoprotein IIbβ monoclonal antibody and FITC-rabbit antimouse IgG fluoresce strongly over background (platelets plus FITC-rabbit antimouse IgG alone). The labeling pattern of the antibody was uniform over the surface of the platelet as would be expected for a labeled major membrane component that is evenly dispersed (data not shown). A similar labeling pattern was observed when the platelets were reacted with heterologous antiglycoprotein IIb and IIIa antibody and FITC-goat antirabbit antibody.

To identify platelets on the basis of their characteristic cell size, unlabeled platelets in plasma were subjected to flow cytometric analysis on the Coulter EPICS V cytometer. Fifty thousand cells were analyzed simultaneously for size by low-angle log light scatter measurements. The platelets had a broad distribution of light-scattering signal which is indicative of size heterogeneity.

Once light scatter parameters for platelets were established, unlabeled and antibody-labeled cells in platelet-rich plasma were analyzed by flow cytofluorimetry. Figure 1 illustrates the fluorescence of the platelet population labeled with both the antiglycoprotein IIbβ and the species-specific FITC-conjugated antibody. Very little green fluorescence was associated with the platelets when only the FITC-conjugated rabbit antimouse antibody was added to the platelet sample indicating that nonspecific binding of this antibody was minimal. Experiments where preimmune mouse IgG was added rather than buffer as the control showed minimal nonspecific binding as well and did not alter the results. The mean fluorescence associated with the platelets in Fig 2 was 13 times that observed in the control experiment. Integration of the fluorescence signal determined that >98% of the cells analyzed as platelets were positive for bound antibody.

Figures 2A and 2B are two-parameter histograms from

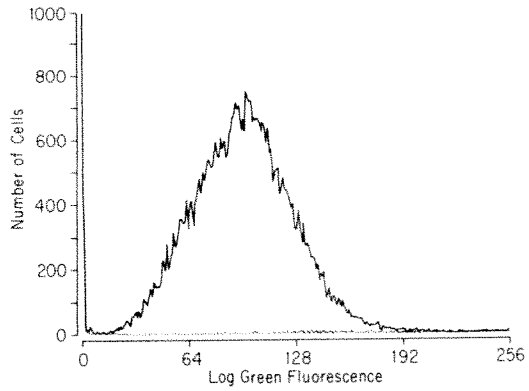

Fig 1. Log green fluorescence intensity of antiglycoprotein IIbβ-treated platelets (——) and platelets treated only with FITC-rabbit antimouse antibody (....). x-axis, log green fluorescence; y-axis, number of cells.

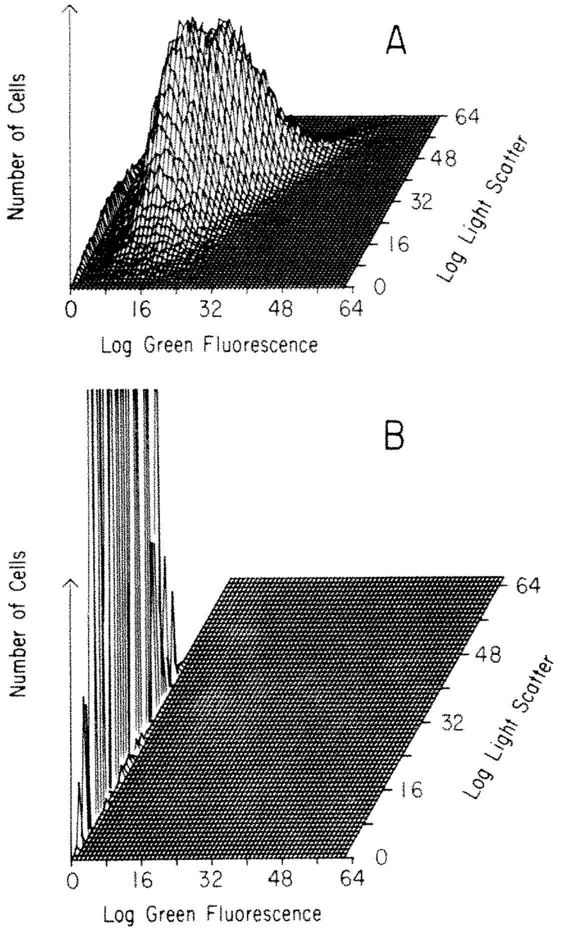

Fig 2. Two-parameter analysis of flow cytofluorimetry of platelets in PRP after addition of 2.5 μg of MoAb18 and FITC-rabbit antimouse IgG (Panel A). The control (Panel B) is of platelets treated in an identical manner except MoAb18 was omitted from the incubation medium. x-axis, log green fluorescence; y-axis, log light scatter; z-axis, number of cells.

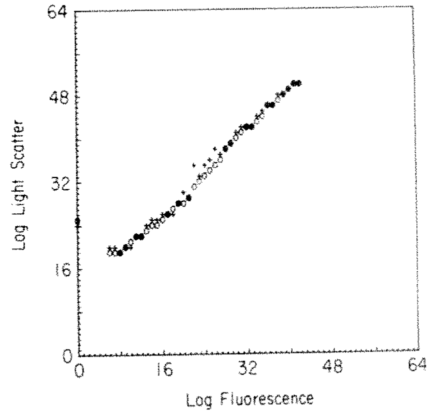

Fig 3. Data in Fig 2, Panel A, replotted as the peak (+) and mean (O) channel of fluorescence for each light scatter channel.

analyses of antibody-labeled cells and unlabeled control cells. In both samples, the platelets had a broad distribution of light scatter similar to that observed in one-parameter light scatter profiles. Platelets treated with mouse IgG or with only the FITC-conjugated rabbit antimouse IgG had no detectable fluorescence over background as all cells were contained in the first three channels of the x-axis. The fluorescence associated with the antibody-treated platelets was significantly greater than background as demonstrated in Fig 2 and was variable depending on the degree of the platelets' light scatter signal. A cursory examination of these data suggests that the larger platelets (those with higher light scatter signal) have a greater mean content of glycoprotein IIbβ than do smaller platelets. This observation is verified by plots of the peak (+) and mean (O) channel of fluorescence for each light scatter channel. As the platelet size increases, the fluorescence (and thus the total content of glycoprotein IIbβ per platelet) increases in a linear relationship (Fig 3).

We next sought to examine normal platelets in whole blood preparations using heterologous antibody directed against glycoproteins IIb and IIIa in a system incorporating unwashed cells, thus avoiding any alterations or artifacts due to cell washing or centrifugation. Platelets were first distinguished from other blood cells on the basis of light scatter. Figure 4 is the log light scatter profile of whole human blood

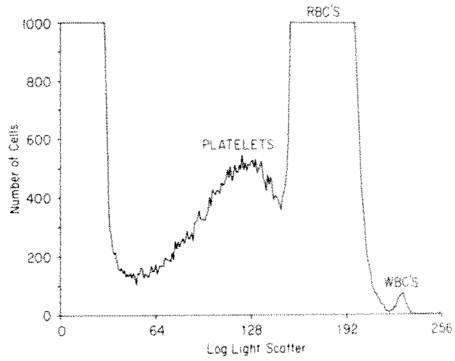

Fig 4. Log light scatter profile of whole human blood-containing platelets (peak 1) and erythrocytes (peak 2) and white blood cells (peak 3). x-axis, log light scatter; y-axis, number of cells.

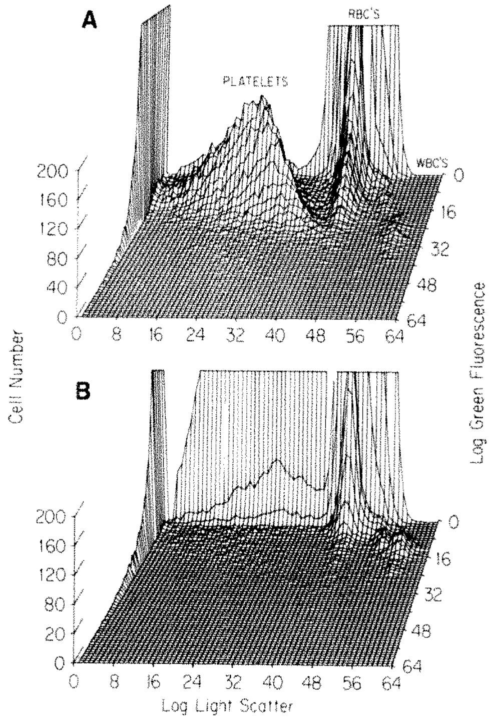

Fig 5. Two-parameter analysis of platelets in whole human blood after addition of anti-GPIIb-IIIa and FITC-conjugated goat antirabbit IgG (Panel A). Platelets in whole blood treated similarly except the anti-GPIIb-IIIa antibody was omitted (Panel B). x-axis, log light scatter; y-axis, number of cells; z-axis, log green fluorescence.

containing platelets, erythrocytes, and white cells. Once the platelets were identified in whole blood, rabbit antiglycoprotein IIb and IIIa antibody and FITC-conjugated goat antirabbit immunoglobulin were added to whole blood as described in Materials and Methods, and the platelets were analyzed on the basis of light scatter and green fluorescence. Fifty thousand platelets were analyzed by collecting data based on log green fluorescence gated on light scatter profile for the platelet population. Figure 5A is the two-parameter histogram of whole blood after addition of antibodies with the corresponding plot of control cells (minus first antibody addition) in Panel B. Platelets were easily distinguishable in whole blood preparations and were specifically labeled by the antiglycoprotein IIb and IIIa antibody. The fluorescence intensity of the antibody-labeled cells was compared to the control group in Fig 6. The log green fluorescence of the antibody-treated platelets was approximately 4 to 5 times higher than that observed with the untreated or control platelet group (plus buffer or normal rabbit IgG instead of the antiglycoprotein IIb and IIIa antibody). Fluorescence of both the red cell and white cell populations appears increased in the antiglycoprotein IIb and IIIa antibody-treated whole blood. While a finite number of platelets were analyzed by collecting data gated on the light scatter profile for platelets, the number of red cells analyzed was different in the control and antibody-treated groups. In the experiment depicted, 56% more red cells were analyzed in the antibody-treated sample than in the corresponding control. Thus, what appears to be an apparent increase in RBC fluorescence is due to increased numbers of red cells being analyzed and not due to specific binding of the antiglycoprotein IIb and IIIa antibody. In contrast, the increased fluorescence observed in the white cell population is due to the detection of bound platelets to monocytes (manuscript in preparation) and is the

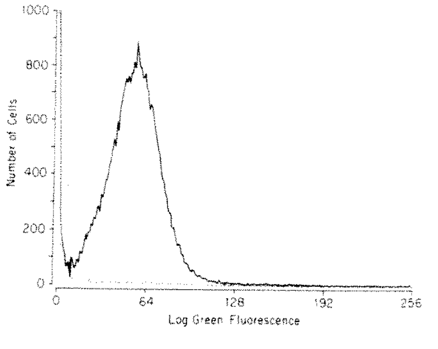

Fig 6. Log green fluorescence intensity of platelets in whole human blood plus anti-GPIIb-IIIa (——) and minus anti-GPIIb-IIIa (....). x-axis, log green fluorescence; y-axis, number of cells.

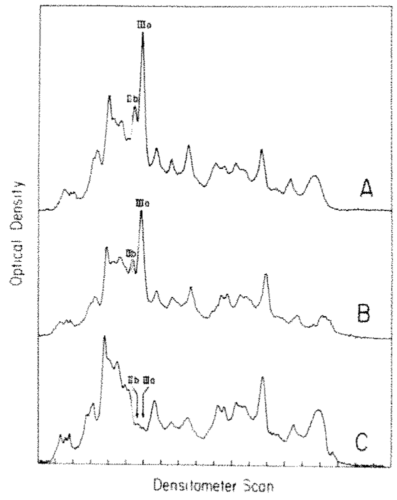

Fig 7. Scanning densitometry of autoradiograms of platelet surface proteins labeled by lactoperoxidase-catalyzed iodination and separated by SDS gel electrophoresis. Scan A, normal donor; Scan B, heterozygote individual; Scan C, GT individual.

Table 1. The Amount of GPIIb-IIIa Determined by Scanning Densitometry of ^{125}I-Labeled Platelet Proteins and by Flow Cytometry Analysis of Relative Fluorescence Intensity

	GPIIb-IIIa Levels	
Individual Analyzed	Scanning Densitometry*	RFI†
Normal	100%	4.0
Heterozygote SJ	73%	3.0
Heterozygote DL	61%	2.6
GT patient RG	6%	1.7
GT patient BL	33%	2.3
GT patient EA	2%	1.4

*Based on the integration of the scan peak corresponding to ^{125}I-labeled platelet GPIIIa relative to platelet actin.

†RFI = relative fluorescence intensity, determined by using the ratio of the mean channel of log green fluorescence intensities of the labeled cells treated with and without anti-GPIIb-IIIa antibody.

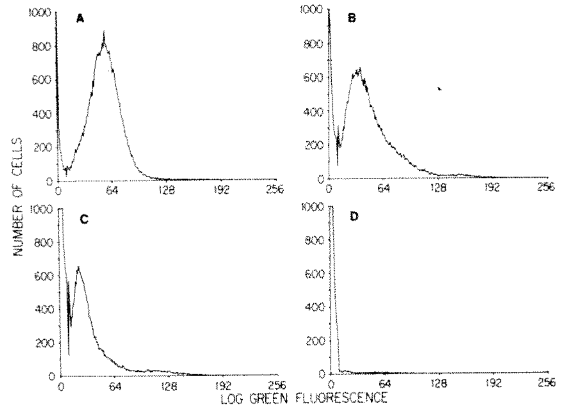

Fig 8. Log green fluorescence intensity profile of platelets in whole human blood labeled by anti-GPIIb-IIIa antibody and FITC-conjugated goat antirabbit IgG. Panel A, normal donor; Panel B, heterozygote SJ; Panel C, Patient RG; Panel D, control sample (anti-GPIIb-IIIa omitted). x-axis, log green fluorescence; y-axis, number of cells.

result of specific binding of the antiglycoprotein IIb and IIIa antibody to these adherent platelets.

This system was then extended to analyze glycoprotein IIb and IIIa content in Glanzmann's thrombasthenic individuals. These values were compared to the levels of glycoproteins IIb and IIIa present in a Glanzmann's thrombasthenic individual's platelets determined by scanning densitometry of autoradiograms of gels of ^{125}I-labeled surface proteins. Two parents of Glanzmann's thrombasthenia patients were included in this study.

Figure 7 is a densitometry scan of radiolabeled platelet surface proteins separated on SDS polyacrylamide gels from patient RG, his parent (SJ), and a representative normal donor. Integration of the peak corresponding to radiolabeled glycoproteins IIb or IIIa of patient RG showed 5.7% of the normal amount of glycoproteins IIb and IIIa (Table 1). The parent had approximately 73% of the normal amount. Patients BL and EA had approximately 33% and 2%, respectively, while DL (parent of BL) had approximately 61% of the normal levels of glycoproteins IIb and IIIa (Table 1).

Platelets were analyzed in whole blood from these individuals by flow cytometric techniques. The histograms of log green fluorescence versus cell number for RG, SJ, and a normal donor are illustrated in Fig 8. Figure 9 is the two-parameter analysis of the same individuals. Individual EA had essentially no detectable levels of glycoprotein IIb and IIIa with a relative fluorescence intensity (RFI) 1.4 times that of untreated platelets, whereas platelets from RG, BL, SJ, and DL and a normal donor had a RFI of 1.7, 2.3, 3.0, and 4.0, respectively (Table 1). Relative fluorescence intensity was determined by using the mean channel of log green fluorescence. These determinations are comparable to glycoprotein IIb and IIIa levels quantitated by autoradiography in that there is a high correlation (r = 0.98) of these levels to the RFI values calculated for each individual (Fig 10). The plot of this data would be expected to intersect at a RFI value of 1.0. The intersection at a slightly higher RFI value is due to the fluorescence associated with nonspecific antibody binding and the inherent autofluorescence of the platelets.

Flow cytometric analysis also permitted us to determine the uniformity of this defect in all individuals tested. The decreased amount of glycoprotein IIb and IIIa was homogeneous within the entire population of analyzed cells confirming that Glanzmann's thrombasthenia affects all platelets in circulation.

DISCUSSION

A variety of biochemical techniques have been used to study the structure of platelet membrane glycoproteins IIb and IIIa and to determine their function in platelet aggrega-

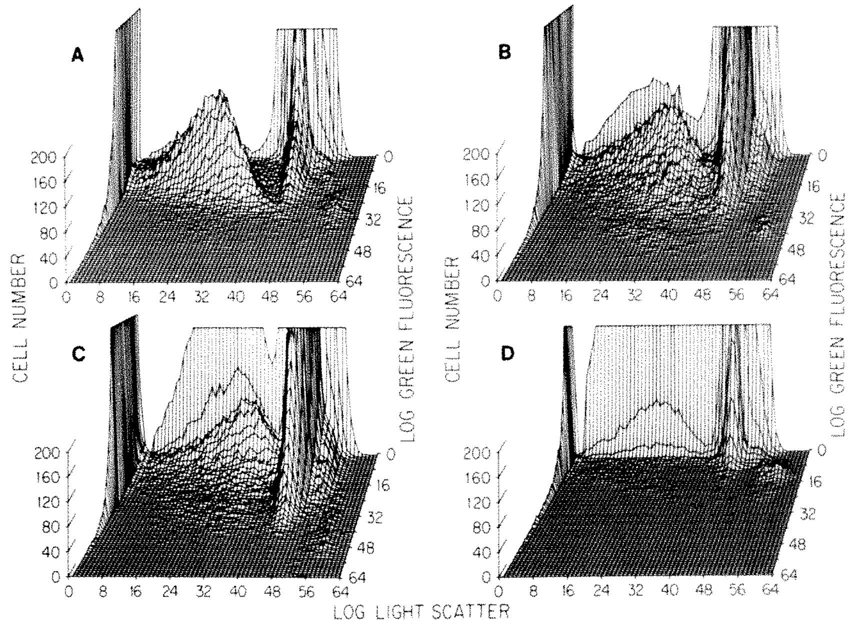

Fig 9. Two-parameter analysis of platelets in whole human blood after addition of anti-GPIIb-IIIa antibody and FITC-conjugated goat antirabbit IgG. Panel A, normal donor; Panel B, heterozygote SJ; Panel C, patient RG; Panel D, control sample (anti-GPIIb-IIIa antibody omitted). x-axis, log light scatter; y-axis, number of cells; z-axis, log green fluorescence.

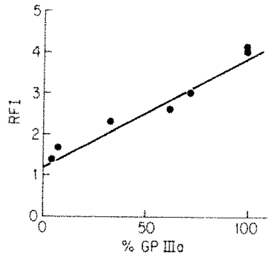

Fig 10. **Quantitation of platelet glycoprotein by Relative Fluorescence Intensity (RFI) of antibody-labeled platelets *v* scanning densitometry of ^{125}I-labeled glycoprotein IIIa. x-axis, % GP IIIa; y-axis, RFI.**

tion. Analysis of these membrane glycoproteins has been based primarily on the development of surface-labeling techniques.[9] Alternative immunochemical methods such as radioimmunoassay (RIA) and crossed immunoelectrophoresis (CIE) have been used to identify and quantify specific platelet surface receptors.[10,11] These methods provide ways of identifying the specific glycoprotein epitopes involved in mediating platelet functions. Studies by Nurden et al[12] have demonstrated that analysis by the immunoblot procedure detected residual amounts of platelet glycoproteins IIb and IIIa which were not initially observed by other methods such as CIE or SDS polyacrylamide gel electrophoresis.

All of these techniques demand a relatively large homogeneous platelet population that requires manipulation through isolation and washing procedures before analysis. Such manipulations may alter the expression or accessibility of surface components and involve considerable preparation time. Recent studies have shown that the association of these glycoproteins is dependent on the buffer and incubation conditions employed. For example, prolonged incubation of platelets or isolated membranes in the presence of EDTA will cause formation of glycoprotein oligomers that cannot be dissociated readily.[13]

We used antibodies that bind to membrane glycoproteins IIb and IIIa to develop methods for analyzing platelets in an unwashed preparation by flow cytometry and to determine if differences in glycoprotein expression could be detected by these methods. We were able to distinguish between Glanzmann's thrombasthenic individuals and carriers for the disorder. We also showed a high correlation between the RFI of the antibody-labeled platelets and the amount of glycoproteins IIb and IIIa analyzed by autoradiography of ^{125}I-labeled platelet proteins.

Platelets in platelet-rich plasma and whole blood were identified by their low-angle log light scatter profile. Although in whole blood preparations there was some overlap in the light scatter distribution of erythrocytes and platelets, erythrocytes can be gated out for platelet analysis or specific probes for platelets can be used in conjunction with their light scatter characteristics. Therefore, flow cytometry is an excellent technique for analyzing platelet surface proteins on a single cell basis. While clinically diagnosed Glanzmann's thrombasthenia patients have variable amounts of glycoproteins IIb and IIIa on their platelet membrane surface, we found that all platelets from each of these individuals were affected equally and that this disorder is not due to distinct populations of circulating platelets. Evaluation of platelets at the single cell levels by flow cytometry would be particularly useful for analysis of acquired platelet disorders such as paroxysmal nocturnal hemoglobinuria (PNH) where distinct platelet populations may occur.[14]

Other investigators have reported using flow cytometry to examine platelet-specific proteins. Adelman and coworkers examined glycoprotein Ib content in washed and formaldehyde-treated human platelets.[15] Johnston and coworkers[16] recently reported their use of flow cytometric techniques to study the effect of diamide and iodoacetamide on the expression of platelet glycoproteins and on platelet aggregation. Their data support our earlier findings[17] that flow cytometry is an efficient method to examine platelet membrane components.

Flow cytometry is also suitable for clinical analysis of platelet surface defects. This technique eliminates the use of radiolabeling techniques and can be performed in conjunction with other surface marker studies. Flow cytometric analysis provides a reliable index of the relative quantity of glycoproteins IIb and IIIa on the platelet membrane. We have demonstrated with our studies of four individuals with various levels of these glycoproteins that the RFI values are comparable with the percentages of glycoproteins IIb and IIIa determined by autoradiography. This method for examining the expression of platelet surface antigens has clear advantages in that platelets may be studied with little or no manipulation and requires only μL quantities of material for analysis. Analyzing platelets in platelet-rich plasma or whole blood provides a milieu most like the in vivo situation. Therefore, the expression of specific epitopes in resting platelets versus activated platelets may be more rigorously and accurately examined by flow cytometric methods.

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DDAVP Infusion in Five Patients With Type Ia Glycogen Storage Disease and Associated Correction of Prolonged Bleeding Times

By Gerald E. Marti, Margaret E. Rick, James Sidbury, and Harvey R. Gralnick

Five patients with glycogen storage disease type I (GSD-I) were evaluated for a bleeding diathesis and subsequently were given an infusion of 1-deamino-8-D-arginine vasopressin (DDAVP). Although platelet counts were normal or slightly elevated, the baseline template bleeding times were prolonged in four of the patients. Prothrombin times and activated partial thromboplastin times were normal, while ADP- and epinephrine-induced platelet aggregations were absent in the three patients tested. Ristocetin- and collagen-induced platelet aggregations were abnormal. Laurell and immunoradiometric determinations of the factor VIII-related antigen (vWf antigen) were decreased. Glyoxyl agarose gel electrophoresis of the patients' plasma revealed abnormal multimer patterns in four of the five patients. After the DDAVP infusion the platelet aggregation abnormalities persisted; however, the bleeding time and the von Willebrand antigen and activity corrected. We conclude that GSD-Ia patients may have a metabolically acquired form of von Willebrand's syndrome as well as an acquired intrinsic platelet defect, and that DDAVP may be useful in the management of bleeding in these patients.

GLYCOGEN storage disease, Type I (GSD-Ia), formerly known as von Gierkie's disease, is an autosomal recessive disease with a deficiency of glucose-6-phosphatase in the liver, kidney, and intestine.[1] It is characterized by hepatomegaly, hypoglycemia, lactic acidosis, hyperlipidemia, hyperuricemia, and a bleeding diathesis. The bleeding diathesis is associated with a prolonged bleeding time and a qualitative platelet defect, while clinically it is manifested by epistaxis and bleeding after invasive procedures or trauma. We have studied the effects of an infusion of 1-deamino-8-D-arginine vasopressin (DDAVP) in five patients with GSD-Ia and show that DDAVP consistently normalizes the prolonged bleeding time and corrects the abnormal values of the factor VIII complex and vWf activities.

MATERIALS AND METHODS

Patients. Five patients with GSD-Ia, previously diagnosed by liver biopsy and enzyme analysis, were admitted to the Clinical Center, NIH, for routine evaluation. Bleeding histories and routine laboratory studies were remarkably similar; all had a history of bruising, epistaxis, bleeding with surgical and dental procedures, and a prolonged bleeding time (Table 1). There were no reported incidences of increased infections.

Coagulation assays. Blood (10 mL) was collected into polypropylene tubes (17 × 100 mm, Falcon) containing 0.1 mL of sodium citrate (32% solution). The tubes were placed on ice and plasma obtained by centrifugation for 10 minutes at 2,000 g at 4 °C. Aliquots were stored frozen at −70 °C.

Prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), and fibrinogen were determined by previously described methods.[2] Factor VIII coagulant activity (VIII:C) was measured in a one-stage assay, factor VIII coagulant antigen (VIII:CAg) by radioimmunoassay,[3] factor VIII-related antigen or vWf antigen by both Laurell and radioimmunoassay,[4] and ristocetin cofactor activity by macroscopic agglutination of fixed platelets.[5] Von Willebrand factor (vWf) multimeric organization was assessed by glyoxyl agarose electrophoresis and autoradiography.[6] Platelet aggregation with patients' platelet-rich plasma was performed with a lumi aggregometer[7] using ADP (3.8 to 16.0 μmol/L), epinephrine (7.5 to 22 μmol/L), collagen (226 μg/mL), thrombin (0.24 U/mL), and ristocetin (0.43 to 1.74 mg/mL) as agonists. Bleeding times were determined using a hand-held, spring-loaded Simplate bleeding time device (General Diagnostics, Morris Plains, NJ).

Table 1. Clinical Summary of GSD, Type I Patients Receiving DDAVP

Patient (age/sex)	Bleeding History	Simplate Bleeding Time (min)	Platelet Count (× $10^3/\mu$L)	Aggregation Studies	PT (sec)	aPTT (sec)	TT (sec)	Fibrinogen (mg/dL)
LRT (22/f)	Severe epistaxis in childhood gum bleeding bleeding with minor surgery	>18	318	abnormal*	12.2	36.5	30.4	502
RSB (26/m)	epistaxis bleeding after dental surgery bleeding with trauma	10.5	384	abnormal*	12.7	36.3	37.2	1,072
MPS (20/m)	epistaxis gum bleeding bleeding with trauma	15	365	abnormal*	13.2	31.4	32.4	570
DS (26/f)	bleeding with loss of deciduous teeth	>18	330	ND	11.4	31.7	28.4	544
JMK (3.5/m)	severe epistaxis	9	570	ND	13.5	38.0	30.5	390
Normal ranges		2.3–9.5	145–364		10.8–13.2	26.8–38.8	26–35	165–335

*See text for specific abnormalities of ADP, epinephrine, collagen- and ristocetin-mediated platelet aggregation.

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DDAVP infusion. A single dose of DDAVP (0.3 μg/kg) was infused over 30 minutes in 50 mL of normal saline. A preinfusion baseline bleeding time and citrated blood sample were obtained from a distal site through an indwelling butterfly needle after discarding the initial 3 to 5 mL of blood. The bleeding time was performed and blood samples were again obtained at 30 minutes, one hour, two hours, and four hours following the infusion. Body weight, serum sodium, hematocrit, and platelet count were determined within 24 hours of completing the infusion. Consent was obtained from patients and/or parents after they were informed of the real and potential risks of this study.

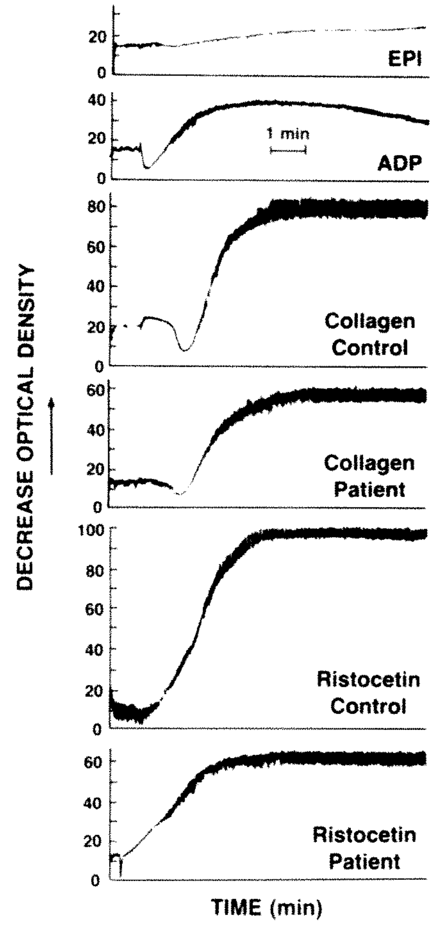

Fig 1. Representative platelet aggregation of PRP from a patient with GSD-Ia. In each experiment 400 μL of patient or normal platelet-rich plasma (200,000 platelets/μL) was incubated for three minutes at 37 °C prior to addition of the platelet agonist; it was stirred at 900 rpm. ATP release was measured by a leuciferase-leuciferin reaction; these tracings are not shown in the figure. Upper Panel: Epinephrine-induced platelet aggregation of the patient's PRP. Epinephrine at concentrations ranging from 7.5 to 21.9 μmol/L did not induce a second wave of platelet aggregation nor was there release of ATP. The control PRP (not shown) showed two waves of aggregation and release of ATP. The second panel is the ADP-induced platelet aggregation of the patient's PRP (ADP at 3.8 to 21.6 μmol/L). A single wave followed by disaggregation was observed. There was no ATP release. The normal PRP (not shown) had two waves of aggregation and released ATP at 4.5 μmol/L ADP. Panels 3 and 4 show the collagen-induced platelet aggregation of the control and the patient. The initial slope of aggregation of the control was much greater than the patient. Maximum collagen-induced aggregation was seen with both patient and control PRP at 242 μg/mL of collagen final concentration. The ristocetin-induced platelet aggregation of the control and patient is shown in the last two panels. The ristocetin concentration was 1.30 mg/mL and at this concentration the initial slope of the patient's PRP was reduced as was the total extent of aggregation. The initial slope of the normal control was 98 mm/min and that of the patient was 33 mm/min. Similar aggregation findings were seen in the other patients studied.

RESULTS

The five patients evaluated in this report all had clinical histories, physical findings, and laboratory values consistent with GSD-Ia and the diagnosis had been confirmed by liver biopsy and enzyme analysis. All five patients had histories of abnormal bleeding which was usually recognized by the age of 2 years and was most often reported as easy bruising and frequent severe nosebleeds. These symptoms became less pronounced by the time the patients became adolescents. Platelet counts were normal or slightly elevated in all patients, but four of five had prolonged template bleeding times (Table 1). The patients were in good metabolic control on multiple cornstarch feedings.

Initial coagulation parameters (PT, aPTT, TT) were normal in all patients except for elevations of fibrinogen. In patient RSB this had been noted five years previously and had been evaluated on numerous occasions by immunologic, biochemical, and chronometric methods; the results were concordant in all three assays. Of interest, this patient has a filling defect on a liver-spleen scan that is thought to be compatible with a benign adenoma.

All three patients tested had abnormal platelet aggregation with ADP and epinephrine. Little or no aggregation was noted with epinephrine while the secondary wave was absent with ADP. Two of the three patients (MPS, RSB) showed no response to low concentrations of ristocetin and had a decreased initial slope at all concentrations of ristocetin and collagen. Aggregation studies repeated after the DDAVP infusion showed no change. Figure 1 is a representative platelet aggregation study.

The responses in the bleeding times of four of five patients to DDAVP are shown in Fig 2. Of the five patients tested, all except one (JMK) showed a decrease in the baseline preinfusion bleeding time to normal. JMK's baseline bleeding time was nine minutes, and a one-hour post-DDAVP infusion bleeding time was eight minutes. By four hours post-DDAVP infusion, the bleeding time had returned to preinfusion values in all five patients. The effects of DDAVP upon VIII:C, VIII R:Ag, and vWf parameters are summarized in Tables 2 and 3. In general, there is a 2- to 4-fold increase in

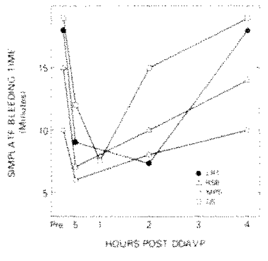

Fig 2. Summary of Simplate bleeding times in four patients with GSD-Ia before and after an infusion of DDAVP.

Table 2. Summary of Percent VIII:C (Coagulant Activity) and VIII:CAg (U/mL) Before and After DDAVP*

Patient	LRT		RSB		MPS		DS		JMK	
	VIII:C (%)	VIII:CAg (%)	VIII:C (%)	VIII:CAg (%)	VIII:C (%)	VIII:CAg (%)	VIII:C (%)	VIII:CAg (%)	VIII:C (%)	VIII:CAg (%)
Baseline (pre)	94	72	104	155	72	62	78	—†	128	—
30′	232	213	316	462	280‡	420	143	122	—	—
1 hour	119	104	—	—	—	—	136	124	320	95
2 hours	113	85	—	—	141	210	82	102	212	146
4 hours	97	85	175	342	103	100	69	—	162	124

*VIII:C = VIII coagulant activity; VIII:CAg = VIII coagulant antigen.
†not done.
‡Sample obtained at 45′.

all parameters evaluated. As was seen with the bleeding times, measurements of VIII:C and VIII R:Ag remained slightly elevated or had returned to baseline within the four hours following the DDAVP infusion.

Baseline vWf activity and VIII R:Ag were abnormal in three patients (LRT, DS, JMK; Table 3). In addition, plasma vWf multimeric patterns on glyoxyl agarose gel electrophoresis showed an abnormal pattern in four of the five patients. The patterns were characterized by a diffuse decrease of all multimers (JMK, moderate; DS, mild), or an apparent decrease in only the larger multimers (RSB, MPS). All five patients showed an apparent increase of the larger forms in their multimeric patterns following the DDAVP infusion, and on occasion a doublet was noted (seen best in the fastest migrating multimer). A composite autoradiogram is shown in Fig 3.

Table 3. Summary of Ristocetin Cofactor Activity and Factor VIII-Related Antigen (measured by both Laurell and RIA) Before and After DDAVP Infusion

Patient	Ristocetin Cofactor Activity (%)	Factor VIII-Related Antigen	
		Laurell (%)	RIA (%)
LRT			
Pre	31	36	—
30′	113	82	145
1 hr	—	—	67
2 hr	64	48	58
4 hr	—	—	74
Repeat sample at 6 mo	47	42	46
RSB			
Pre	95	128	—
30′	215	228	197
4 hr	182	228	183
MPS			
Pre	57	61	104
45′	246	213	197
4 hr	163	183	183
DS			
Pre	26	24	33
30′	—	—	102
1 hr	89	103	107
2 hr	83	88	91
4 hr	29	56	63
JMK*			
Pre	8	20	15
1 hr	108	123	65
2 hr	116	188	156
4 hr	110	178	90
Repeat sample at 6 mo	45	32	29

*Values for JMK's younger sister were ristocetin factor activity 85%, and for the Laurell and RIA determinations 48% and 34%, respectively.

DDAVP side-effects. During the DDAVP infusion four of the five patients showed facial flushing which they were unaware of, and one patient complained of vague abdominal cramping. No other side-effects were noted. There was no change in weight, hematocrit, or platelet count.

DISCUSSION

The occurrence of a bleeding tendency has been widely recognized in patients with GSD-Ia. It is usually recognized in early infancy and is characterized by easy bruising, severe nosebleeds, and prolonged bleeding following minor trauma, surgical or dental procedures.[8] However, bleeding with liver biopsy is unusual. Most investigators report a prolongation of the bleeding time despite normal platelet counts.[9-17] Lelong et al first demonstrated a qualitative platelet defect, and documented decreased prothrombin consumption and a retardation of platelet thromboplastin generation.[9] Although it was initially thought that platelets from patients with GSD-I had higher levels of glycogen and a deficiency of glucose-6-phosphatase, this observation has not been confirmed.[13,16]

We have studied five patients with type GSD-Ia. We found abnormal platelet aggregation, prolonged bleeding times, reduced VIII:RAg and vWf activity, and abnormal vWf multimer plasma patterns. In the present study, the VIII:C activity was normal in all individuals tested (Table 2). The ristocetin cofactor activity and VIII R:Ag levels were abnormally low in three of the patients (LRT, DS, JMK). The abnormalities in the multimer patterns showed either a diffuse decrease (DS, JMK) or an additional decrease in the largest multimers (RSB, MPS). These data indicate that some patients with GSD-Ia may have an acquired von Willebrand syndrome or a syndrome that mimics vWd.

Our five patients with GSD-Ia were given DDAVP intravenously. During the ensuing four hours after infusion, we observed an increase to normal levels of VIIIR:Ag in all patients and a correction of the bleeding time in four of five patients. The multimer patterns of plasma vWf showed an increase in all multimers and the appearance of larger multimers in those plasmas that were previously deficient.

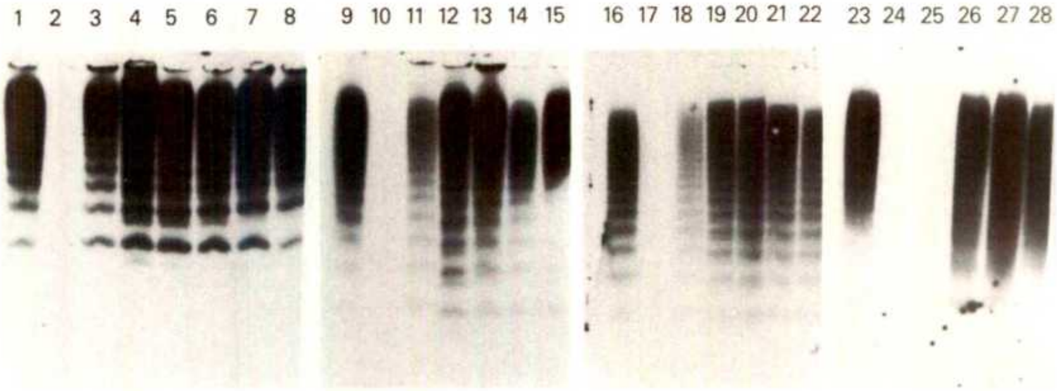

Fig 3. Composite summary of glyoxyl agarose gel electrophoresis autoradiographs before and after an infusion of DDAVP in five patients with GSD-Ia. Lanes 1, 8, 9, 16, and 23 contain normal pooled plasma. Lanes 2, 10, 17, and 24 contain plasma from an individual with severe von Willebrand's disease. Lanes 3 through 7 are pre-DDAVP (baseline), post-DDAVP 30', one hour, two hours and four hours, respectively, in patient LRT. Lanes 11 through 13 are pre-DDAVP, post-DDAVP 45' and two hours, respectively, in patient MPS. Lanes 14 and 15 are pre-DDAVP and post-DDAVP 30' in patient RSB. Lanes 18 through 22 are pre-DDAVP, post-DDAVP 30', one hour, and two hours, respectively, in patient DS. Lanes 25 through 28 are pre-DDAVP, post-DDAVP one hour, two hours, and four hours, respectively, in patient JMK. Although no radioactivity is appreciated in lane 25 of the photograph, reduced multimers were seen on the autoradiograph.

DDAVP presumably causes the release of vWf multimers including high molecular weight multimers from endothelium and/or other storage sites.[18,19] In patients with type I and type IIa vWd this is associated with a transient shortening or normalization of the bleeding time, and in vitro perfusion studies have shown increased platelet adherence and platelet aggregate formation.[20] The five patients examined in this report all responded to DDAVP in a manner similar to that seen in patients with vWd, hemophilia A, uremia, and dysfunctional platelet syndromes.[21-28]

In vitro platelet function studies have demonstrated the absence of a secondary wave in ADP- and epinephrine-induced platelet aggregation, while with collagen and ristocetin the response is diminished.[13-15] Platelet adenine nucleotide levels may be normal or reduced and the defective release of ADP remains to be confirmed.[13-15] Reports of mixing experiments involving GSD-I plasma and its effect upon normal platelet aggregation are contradictory.[13,14,17] Early attempts to characterize the mechanism of the platelet dysfunction examined the relationship between the prolonged bleeding times and metabolic abnormalities: hypoglycemia, lactic acidosis, hyperuricemia, and hyperlipidemia. Several investigators[13-15,29] have shown that infusions of glucose, intravenous hyperalimentation, and total parenteral hyperalimentation can lead to correction of the bleeding times and in vitro platelet function. However, it would appear that several days (10 to 12) of this therapy are required before normalization occurs.[14] Portacaval shunting can result in the long-term correction of these abnormalities.[14,24]

Kao et al reported impaired ristocetin-induced platelet aggregation in six children with GSD-Ia.[17] Studies with washed platelets indicated a plasma abnormality rather than an intrinsic platelet defect, and binding studies revealed a normal number of VIII:RAg receptor sites on GSD-Ia platelets. Further studies suggested that the fibrinogen from GSD-Ia plasma was inhibiting ristocetin-induced agglutination in these patients. A posttranslational modification of the carbohydrate in fibrinogen could occur in plasma of GSD-Ia patients which might account for this phenomenon[30]; further analysis of our patients' fibrinogens is being carried out.

The present study confirms previous reports of abnormal platelet aggregation in conjunction with prolonged bleeding times in patients with GSD-Ia. The data also indicate a probable high incidence of decreased vWf levels and activity in these patients. Regardless of the nature of the defect(s), our data demonstrate that GSD-Ia patients responded to DDAVP in a predictable manner, and suggest that DDAVP may be efficacious in the treatment of bleeding episodes or treatment prior to minor surgical procedures in these patients.

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Synthesis of Eosinophil-Associated Enzymes in HL-60 Promyelocytic Leukemia Cells

By Steven A. Fischkoff, Glenn E. Brown, and Avrom Pollak

Eosinophils derived from HL-60 cells share many of the abnormalities of granule histochemistry and morphology frequently seen in eosinophils of patients with certain malignancies, especially those seen in acute myelomonocytic leukemia with abnormal eosinophils (FAB class M4eo). In order to understand the pathogenesis of these abnormalities, four enzymes, characteristic of the eosinophil, were studied in HL-60 promyelocytic leukemia cells at various stages of eosinophilic differentiation. Using biochemical and ultrahistochemical techniques, the following differences from normal eosinophil development were demonstrated. First, both myeloperoxidase and eosinophil peroxidase coexisted in the population of maturing HL-60 eosinophils. Second, the granules formed from the condensation of material in vacuoles which were derived from dilated segments of the endoplasmic reticulum; the role of the Golgi apparatus in processing of peroxidase appeared minimal. Third, low levels of lysophospholipase and arylsulfatase were present in the cells compared to normal eosinophils. Finally, crystallizations resembling precursor structures of Auer rods appeared in the granules of about 5% of the cells. These findings suggest that several disorders of the control of protein synthesis and processing exist in HL-60 eosinophils which may be responsible for the abnormal granule morphology and histochemistry.

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MORPHOLOGICALLY and histochemically abnormal eosinophils have been described in a variety of disease states, notably lymphoid and myeloid malignancies.[1-4] Alterations in the pathways by which granule proteins and enzymes are processed and packaged in myeloid precursors have been documented in certain of these disorders.[1,5] For example, little stainable peroxidase activity could be demonstrated in the Golgi apparatus in myeloid precursors from patients with acute nonlymphocytic leukemia despite abundant stained material in the endoplasmic reticulum and granules.[5,6] In normal eosinophils the Golgi apparatus is intrinsically involved in peroxidase processing.[7] Because the pathways for protein synthesis and processing have been studied in detail in normal eosinophil precursors,[7,8] careful examination of these pathways in the abnormal immature eosinophils should clarify the pathogenesis of the granular abnormalities.

Our finding that the HL-60 cell line appears to be a model for a recently recognized subset of acute myelomonocytic leukemia (FAB class M4eo), characterized by the presence of dysplastic bone marrow eosinophils and chromosomal break at 16q22, provides an opportunity to study these pathways in a controlled, in vitro model system.[9] In particular, both the HL-60 eosinophils and the bone marrow eosinophils of these patients share the following features of their granules: they are frequently large in size and irregular in shape; they stain variably from red to blue with the Wright stain, even in the same cell; crystalloids are absent; and the Periodic acid-Schiff (PAS) and chloroacetate esterase reactions are positive (normal eosinophil granules are negative for both).[4,9] Since similar granule abnormalities have also been observed in eosinophils of patients with other cancers, the underlying mechanistic alterations present in acute nonlymphocytic leukemia with abnormal eosinophils (ANLL-M4eo)[2] patients may also be operative in these other diseases with abnormal eosinophil granules. Therefore, characterization of the pathway of granule protein processing in HL-60 cells induced to differentiate to eosinophils, and comparison to that described in normal eosinophils should lead to an improved understanding of the pathogenesis of the abnormal eosinophil granules in ANLL-M4eo and possibly of other malignant diseases.

MATERIALS AND METHODS

Cell culture and inductions. HL-60 cells were cultured in RPMI-1640 with 10% heat-inactivated fetal calf serum (K.C. Biological, Lenexa, Kan) and penicillin and streptomycin (50 IU/mL and 50 ug/mL, respectively, Flow Laboratories, Rockville, Md; "complete medium") in a humidified atmosphere of 5% carbon dioxide. Cell cultures to be used for eosinophil inductions were grown differently from those used for neutrophil inductions.[10] The eosinophil-destined cultures were subcultured on Fridays to a concentration of 5×10^5 cells/mL with complete medium adjusted to pH 7.6 to 7.7 with NaOH. On Mondays, an equal volume of this medium was added. On Tuesdays, Wednesdays, and Thursdays the flasks were removed from the incubators until the pH rose to 7.6 (approximately two hours for T-75 flasks; Corning, Corning, NY). After culturing in this fashion for at least two months, eosinophilic differentiation was induced by suspending these cells at 5×10^5 cells/mL in complete medium at pH 7.4, adding 0.5 mmol/L butyric acid (as a 0.5 mol/L stock solution in ethanol, Sigma, St Louis) and incubating the flasks for seven days without further attempts at controlling medium pH. Cells used for neutrophil inductions were subcultured on Fridays to a concentration of 5×10^5 cells/mL with complete medium adjusted to pH 7.2 to 7.3 and fed with an equal volume of complete medium on Mondays. No further measures were performed to control pH. For neutrophil inductions, these cells were suspended at 5×10^5 cells/mL in complete medium containing 176 mmol/L dimethylsulfoxide (DMSO; Aldrich Gold Label, Aldrich, Milwaukee), and the cultures incubated for seven days. The extent of differentiation was assessed by differential counts of Wright-stained cytospin preparations.

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Electron microscopy. Cells were fixed for one hour in either 4% formaldehyde-1% glutaraldehyde in phosphate buffered saline (for peroxidase staining) or 2% glutaraldehyde in cacodylate buffer (for acid phosphatase, arylsulfatase, and lysophospholipase staining). Peroxidase activity was localized using 3:3′-diaminobenzidine (Sigma; see reference 11). Acid phosphatase activity was localized with β-glycerophosphate or p-nitrophenyl phosphate (Sigma; see reference 12). Arylsulfatase activity was localized with p-nitrocatechol sulfate (Aldrich; see reference 13) and lysophospholipase with lysophosphatidylcholine (Sigma; see reference 14). Cell pellets were embedded, sectioned, and examined with a JEOL 100CX electron microscope.

Enzyme extractions and assays. For each assay, 1×10^7 cells were washed and suspended in 1 mL of lysis buffer and sonicated for three minutes at Power Level 6 in a bath-type sonicator (Heat Systems W-225R). The compositions of the lysis buffers, substrates, and references for assay methods are given in Table 1. Oxidized minus reduced difference spectra for myeloperoxidase and eosinophil peroxidase were obtained using sodium dithionite as a reductant in an Aminco DW-2 double-beam spectrophotometer.[18] To examine the isoenzymes of acid phosphatase, the cell pellets were suspended in 0.2% (w/v) 3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate (CHAPS; Sigma) in 0.01 mol/L Tris-HCl, pH 7.4, to a final concentration of 2×10^8 cells/mL. The suspensions were sonicated at a setting of 7 for 40 seconds, followed by pelleting of the debris at 13,000 x g for three minutes at room temperature. Twenty μL samples were subjected to isoelectric focusing on Servalyte Precotes (pH 3 to 10, Serva) using a programmable power supply (Pharmacia ECPS 3000/150) as described by Serva. Activity bands were visualized with α-naphthol phosphate (Sigma) as a substrate and Fast Black K (Sigma) as the coupler.[19] Inclusion of 0.2% CHAPS in the extraction buffer increased the number of bands resolved and their intensities. Analysis of statistical significance was performed via the one-tailed student's *t* test.

RESULTS

Induction of differentiation. Over the course of a seven-day incubation with butyric acid, the HL-60 cells which had been previously cultured at pH 7.6 underwent differentiation to eosinophil-like cells. All induced cultures used for the enzymologic studies below consisted of no less than 50% eosinophils and no more than 20% neutrophils (balance of 100% was undifferentiated cells). Cells treated with DMSO for seven days underwent differentiation to neutrophil-like cells, albeit to a slightly lesser extent than that expected for HL-60 cells previously grown at pH 7.2. These cultures consisted of greater than 50% neutrophils (typically greater than 70%) and no more than 10% eosinophils (balance of 100% was undifferentiated cells). While the population of cells matured steadily in response to butyric acid, there was always cell-to-cell variability in the extent of maturation such that homogeneous populations of eosinophils at any particular level of maturation never existed. Nevertheless, on the electron photomicrographs, there was good morphologic correlation between the degree of nuclear maturation using criteria summarized by Wetzel (margination and condensation of chromatin, presence of nucleoli, nuclear size, and nuclear lobulation; reference 20) and the cytoplasmic changes described below. Thus, it was possible to use nuclear maturation as an internal clock by which to sequence cytoplasmic changes that occur with maturation in a mixed population of maturing cells. This approach has previously been used by Presentey et al.[1]

Peroxidase activity. Peroxidase activity was present in immature HL-60 cells populations and increased approximately 30% over seven days in untreated cultures (Fig 1). There was a steady decrease in enzyme activity per cell in DMSO-treated cultures beginning as early as the first day of culture until 45% of the initial peroxidase activity remained at the end of seven days. However, enzyme activity dropped at 24 to 48 hours and then increased above the baseline levels during the eosinophil induction with butyric acid (Fig 1). Because cell numbers increased in these cultures while enzyme activity per cell increased, new enzyme synthesis must have occurred.

We previously reported the presence of cyanide-resistant peroxidase activity in the HL-60 eosinophils using histochemical methods.[9] This implied that eosinophil peroxidase, which is a different molecular species than myeloperoxidase, was present. In order to more firmly establish which isoenzymes of peroxidase were present in the HL-60 eosinophils, difference spectra with and without reduction by sodium

Table 1. Enzyme Activity in HL-60 Cells in the Undifferentiated State and After Neutrophilic or Eosinophilic Differentiation

	Enzyme Activity†		
Enzyme*	Uninduced	Neutrophilic	Eosinophilic
Peroxidase (total)[15]	$0.398 \pm 7.1 \times 10^{-4}$	0.205 ± 0.088 (0.031)	$0.351 \pm 2.3 \times 10^{-4}$ (0.151)
Acid Phosphatase[15]	0.036 ± 0.045	0.421 ± 0.268 (0.027)	0.589 ± 0.036 (2.5×10^{-5})
Arylsulfatase[16]	0.135 ± 0.014	0.157 ± 0.070 (0.031)	0.235 ± 0.045 (0.011)
Lysophospholipase[17]	20.5 ± 2.4	61.8 ± 31.8 (0.046)	76.3 ± 51.4 (4.9×10^{-3})

HL-60 cells were cultured for seven days at an initial concentration of 5×10^5 cells/mL of complete medium in the presence of no addition (uninduced), 176 mmol/L DMSO (neutrophilic), or 0.5 mmol/L butyric acid (eosinophilic). Cells were harvested, sonicated in the lysis buffers (see footnote), and the extracts assayed for enzyme activity as per the given reference. Values given represent the mean of three values ± SD with the *P* value in parenthesis below.

*Lysis buffers: Peroxidase and acid phosphatase, 0.3% (w/v) Triton X-100 in H_2O; arylsulfatase, H_2O; lysophospholipase, Dulbecco's phosphate-buffered saline.

†Units: Peroxidase, ΔO.D. 560/1 min/10^6 cells; acid phosphatase, ΔO.D.405/2 h/2.5×10^5 cells (nonspecific activity in presence of 2 mmol/L NaF subtracted); arylsulfatase, ΔO.D. 515/1h/10^6 cells; lysophospholipase, counts per minute (above blank)/1 h/10^6 cells.

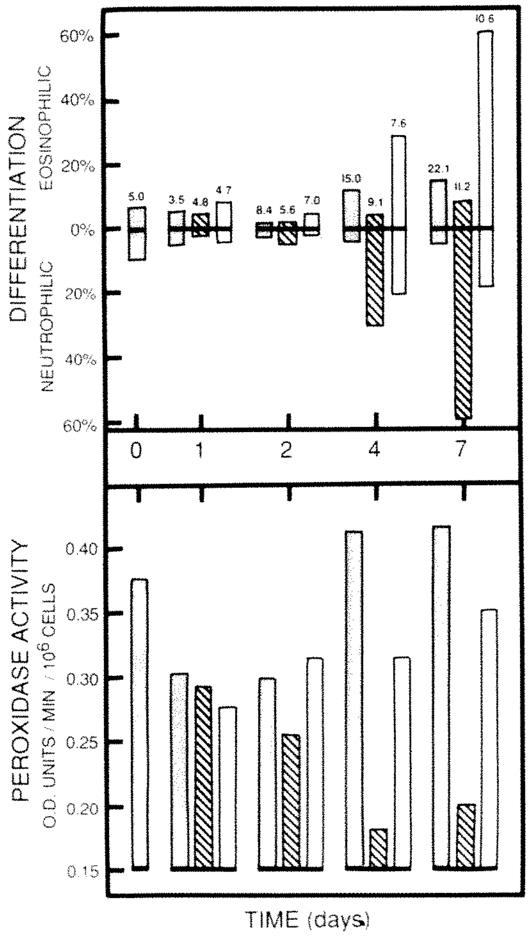

Fig 1. HL-60 cells, previously grown at pH 7.6, were suspended at 5 × 10^5 cells/mL in complete medium in the presence of no added compound (stippled bars), 176 mmol/L DMSO (cross-hatched bars), or 0.5 mmol/L butyric acid (open bars). Aliquots were removed at days 0, 1, 2, 4, and 7 for cell and differential counts (upper panel) and for assay of peroxidase activity (lower panel). The differential counts are presented as upward bars for cells with eosinophil granules and downward bars for neutrophils. Only cells with nuclei in the myelocyte, metamyelocyte, banded, or segmented stage of maturity were counted. Myeloblasts and promyelocytes represent the balance of 100%. The cell counts (× 10^{-5} cells/mL) are shown above the differential count bars. The peroxidase activity is expressed as the change in O.D. units per minute per 10^6 cells. All values represent the mean of 3 determinations (upper panel) or 5 to 6 (lower panel). Standard deviations did not exceed 20% for cells counts or enzyme activities.

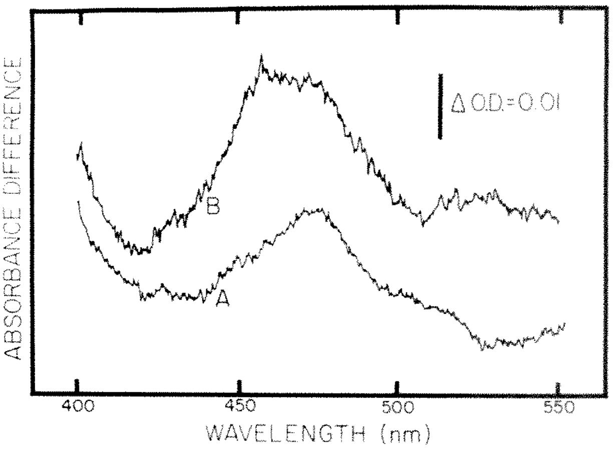

Fig 2. Extracts were prepared from uninduced HL-60 cells (trace A) and HL-60 eosinophils after seven days of culture with 0.5 mmol/L butyric acid (trace B) by sonication of 10^7 cells/mL in 0.3% Triton X-100 in water. Half of each sample was reduced with sodium dithionite and difference spectra obtained.

dithionite were obtained in a double-beam spectrophotometer using extracts before and after induction of eosinophilic differentiation (Fig 2). Uninduced cell extracts yielded a single peak at 474 nm corresponding to myeloperoxidase. HL-60 eosinophil extracts demonstrated an additional peak at 456 nm corresponding to eosinophil peroxidase in addition to the myeloperoxidase peak resulting in a broad plateau.

Peroxidase ultrastructural localization. HL-60 cells grown at pH 7.6 were treated with 0.5 mmol/L butyric acid for seven days, stained for peroxidase activity, and examined via the electron microscope. The least mature HL-60 cells demonstrated peroxidase activity in the endoplasmic reticulum and perinuclear cistern (Fig 3A). These cells lacked the peroxidase-positive primary granules usually seen in immature HL-60 cells grown at lower pH, thus being most similar to the myeloblast. With continued enzyme synthesis, dilatation of the endoplasmic reticulum occurred which filled with a granular, flocculant, peroxidase-positive material (Fig 3B). Vacuoles were found, seeming to arise from more markedly dilated segments of endoplasmic reticulum. These segments were also filled with the peroxidase-containing material. In cells with nuclei at the metamyelocyte stage of maturity (Fig 3C), peroxidase activity was again seen in the endoplasmic reticulum and, to a minor extent, in the Golgi apparatus. The vacuolar dilatations were more extensively developed. The material in the vacuoles underwent condensation, forming granules (Fig 3C, arrow). Vesicular budding of peroxidase-positive material from the Golgi apparatus could not be demonstrated. With continuation of the process of condensation of vacuole material into granules (Fig 3D), less enzyme activity was demonstrable in the endoplasmic reticulum and that in the perinuclear cistern virtually disappeared. The completed granules were not free in the cytoplasm but were membrane bound. The membrane probably represent the wall of the vacuole, now adherent to the granule. Further cellular maturity was associated with disappearance of enzyme activity from the endoplasmic reticulum so that all peroxidase activity was localized to the granules. In approximately 5% of cells, the granule proteins crystallized into randomly oriented tubules and multilammellar structures composed of peroxidase-positive subunits (Fig 4). Dixon et al suggested that such structures in leukemic promyelocytes represented precursors of Auer rods.[21] While elongated structures derived from intergranular fusion were found, fully developed, typical Auer rods were never observed.

Acid phosphatase. The level of acid phosphatase activity increased in response to a seven-day eosinophil induction with butyric acid (Table 1). Increased enzyme activity has been similarly reported with DMSO-induced neutrophil

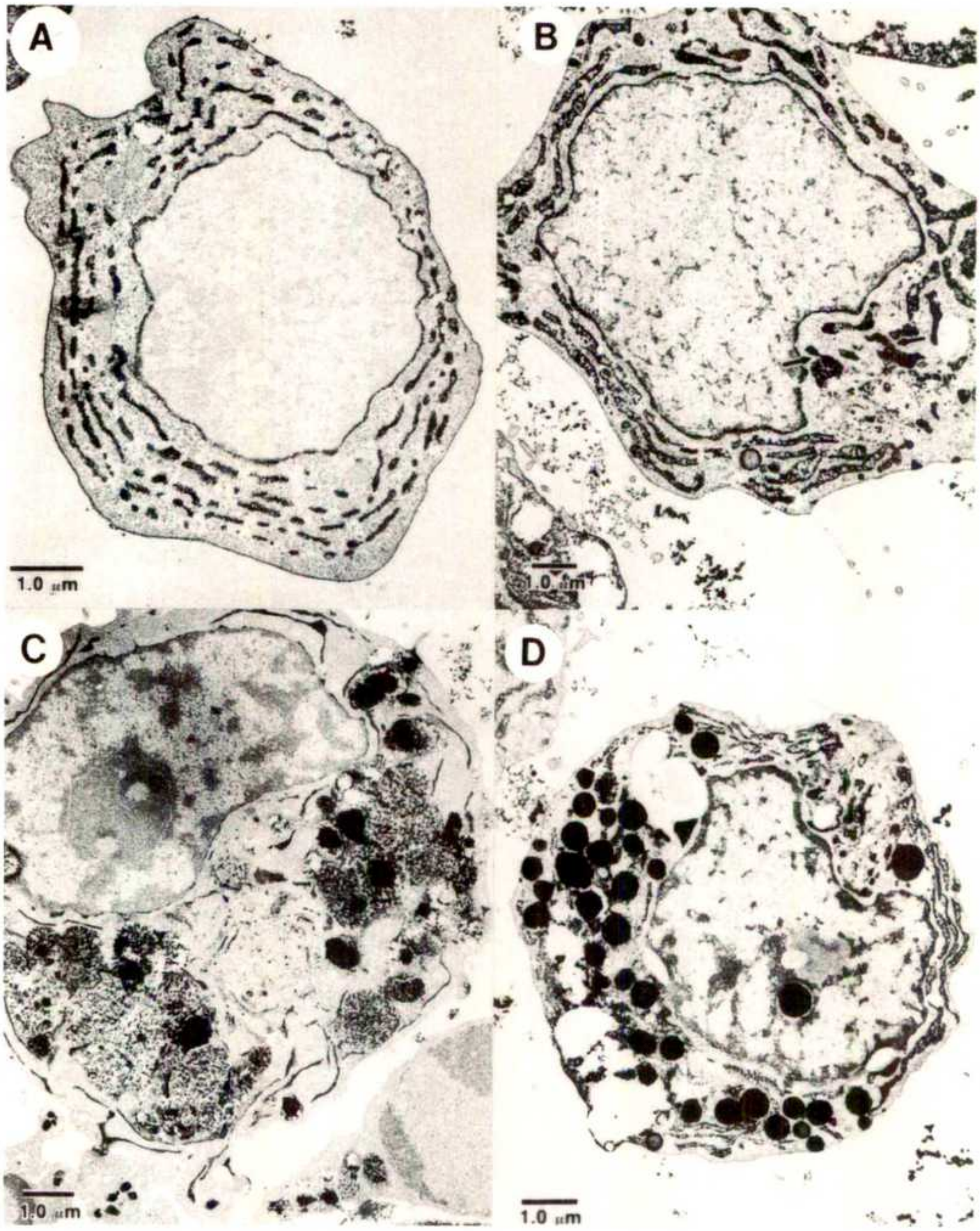

Fig 3. HL-60 cells previously grown at pH 7.6 were cultured for seven days with 0.5 mmol/L butyric acid, fixed, and stained for peroxidase activity. (A) This cell demonstrates the absence of primary granules and peroxidase activity in the endoplasmic reticulum and perinuclear cistern. Bar = 1.0 μm. (B) Dilatation of the endoplasmic reticulum into vacuoles filled with peroxidase-positive material is shown. Arrows point to areas where the vacuoles and endoplasmic reticulum are continuous. Bar = 1.0 μm. (C) Development of granules within vacuoles containing peroxidase-positive material is demonstrated. The Golgi apparatus is poorly stained for peroxidase activity despite easily seen activity in the granules and endoplasmic reticulum. Bar = 1.0 μm. (D) Virtual completion of granule formation has occurred. Enzyme activity has disappeared from the perinuclear cistern, although it persists in the endoplasmic reticulum. Bar = 1.0 μm.

differentiation and 12-O-tetradecanoylphorbol-13-acetate (TPA)–induced macrophage differentiation of the HL-60 cells.[12] Extracts of HL-60 cells, treated for seven days with either DMSO (neutrophil) or butyric acid (eosinophil), were subjected to isoelectric focusing and the gels stained for acid phosphatase activity. Thirteen bands were seen at identical isoelectric points ranging from 4.5 to 6.2. While some differences in intensity were seen between individual corresponding bands, no unique activities were observed in the HL-60 eosinophil extracts, nor were any activities seen in the DMSO-treated samples unrepresented. Some insoluble activity remained at the origin regardless of the use of Triton X-100 and/or CHAPS precluding estimates of the proportion of activity in each band.

Ultrastructure localization of acid phosphatase activity. Unlike peroxidase activity, acid phosphatase activity was not seen in the endoplasmic reticulum or Golgi apparatus of immature HL-60 eosinophils using either β-glycero-

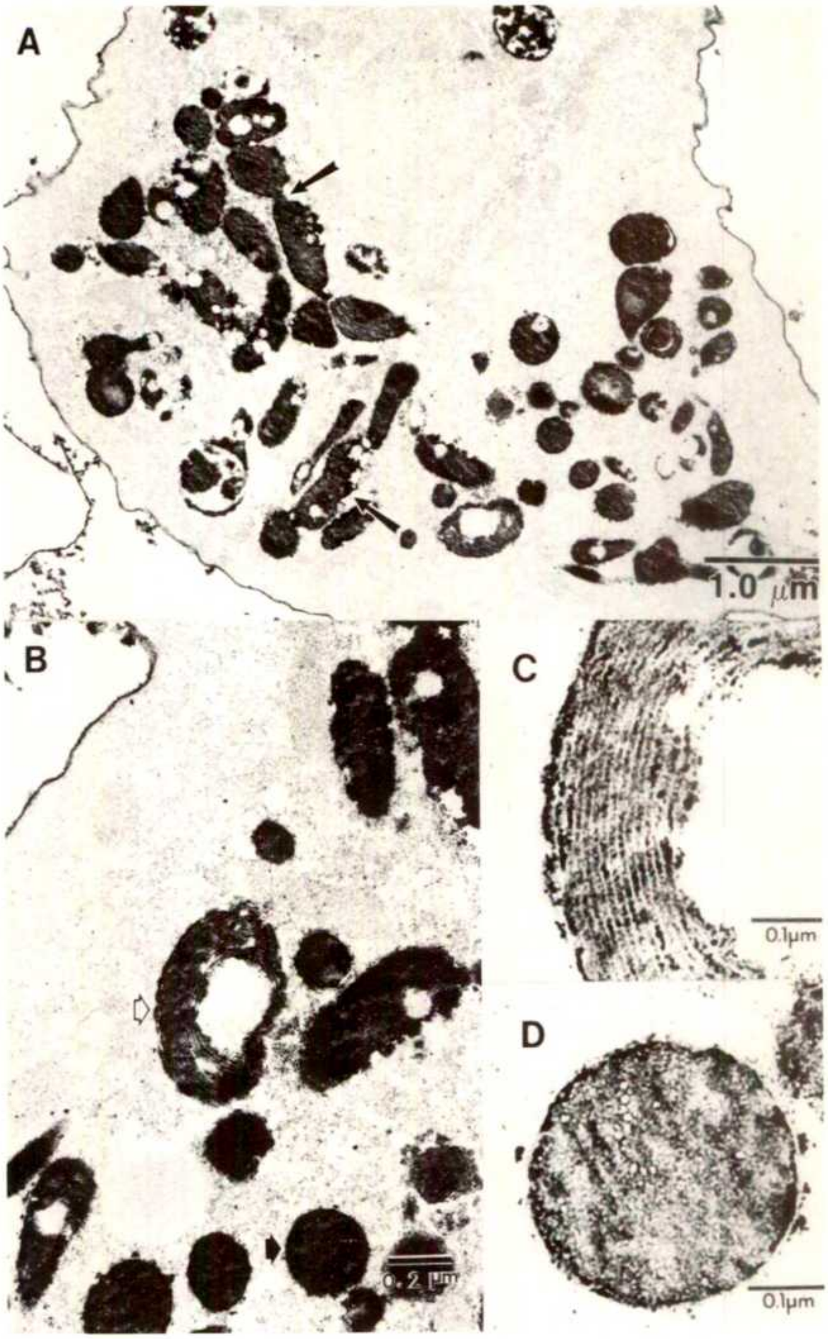

Fig 4. HL-60 cells grown and processed as in Fig 3 demonstrate completion of the process of granule formation and disappearance of peroxidase activity from the endoplasmic reticulum. In this cell, crystallization of granule proteins occurred yielding multilamellar structures and tubules composed of peroxidase-positive subunits. (A) Low magnification view demonstrates intergranular fusion, yielding elongated structures (arrows). Bar = 1.0 µm. (B) Detail of previous micrograph demonstrates peroxidase staining in areas where lamellar structures and tubules are developed. Bar = 0.2 µm. (C) High magnification of granule in B marked with open arrow demonstrates subunits in lamellae. Bar = 1.0 µm. (D) High magnification of granule in B marked with closed arrow demonstrated tubules in crosssection. Bar = 0.1 µm.

phosphate or p-nitrophenyl phosphate as a substrate. However, acid phosphatase–positive flocculant material was also found in many vacuoles and appeared to condense into granules. P-nitrophenyl phosphate, unlike β-glycerophosphate, can be used to demonstrate acid phosphatase activity in extralysosomal sites in mammalian cells.[12] This did not appear to be the case in the HL-60 eosinophils. As previously described for normal eosinophil granules,[22] condensation of acid phosphatase appeared to result in enzyme inactivation so that, often, only a rim of activity could be demonstrated around the granules (Fig 5A). This phenomenon was also seen in granules where the multilamellar structures formed (Fig 5B). Unlike peroxidase activity, in areas where the crystalline structure was poorly formed, enzyme activity was preserved; in areas where it was well formed, enzyme activity was greatly diminished or absent.

Arylsulfatase and lysophopholipase. Both of these enzymes were expressed in the HL-60 neutrophils and eosinophils (Table 1). However, eosinophils in peripheral blood smears from normal individuals and from a patient with hypereosinophilia secondary to a lung tumor showed considerably more intense staining for both enzymes than did HL-60 eosinophils at the light microscopic level. Ultrastructurally, minor deposits representing arylsulfatase activity could be seen at the surface of some granules if the sections were not counterstained. Deposits representing lysophospho-

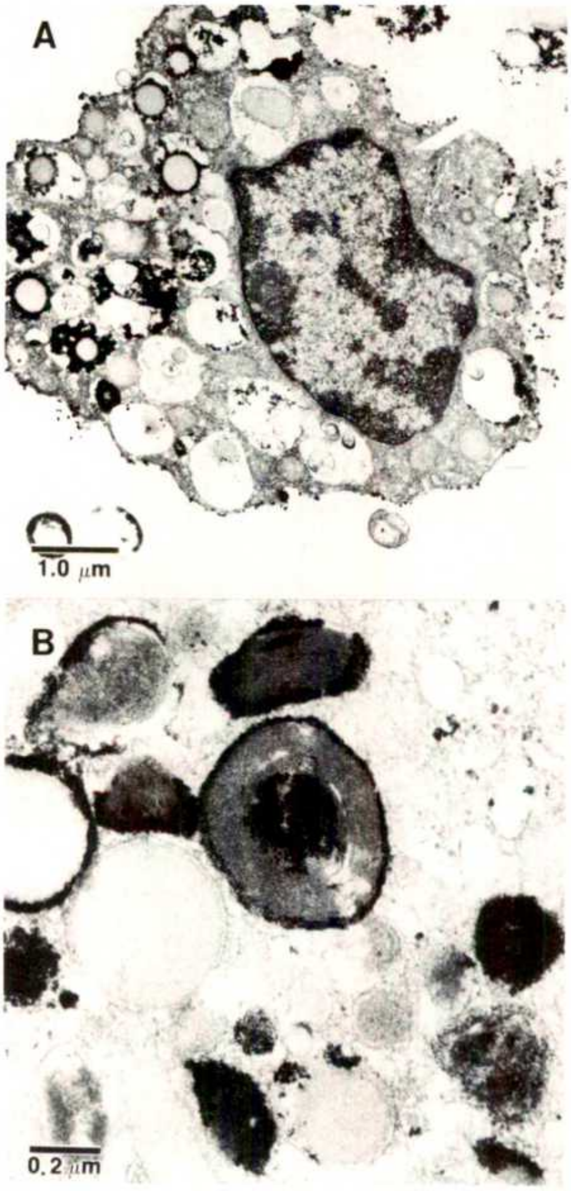

Fig 5. HL-60 cells were grown as in Fig 3, fixed, and stained for acid phosphatase activity using β-glycerophosphate. (A) Acid phosphatase-positive material is seen condensing into granules that no longer demonstrate enzyme activity. Bar = 1.0 μm. (B) Acid phosphatase activity has disappeared from areas undergoing crystallization to multilamellar structures, but is retained in adjacent, poorly crystallized area. Bar = 0.2 μm.

lipase activity were demonstrated in dilated segments of endoplasmic reticulum. However, for both enzymes, the deposits were not consistently found in all the HL-60 eosinophils.

DISCUSSION

In order to define the pathways by which the eosinophil-associated enzymes are expressed in HL-60 cells induced to differentiate to eosinophils, a combined approach was taken. First, the activities of these enzymes were measured in whole cell extracts at different time points during the induction of differentiation. In some cases, isoenzyme studies were performed. Second, enzyme activities were localized at the ultrastructural level using ultrahistochemical techniques at various stages of eosinophil maturity. This latter approach, previously used by others,[1] was made possible by the apparently constant relationship between the degree of maturity of the nucleus based on morphologic criteria and the degree of development of the cytoplasm. Although that relationship was not identical to that reported in normal eosinophil precursors, it was possible to use the nuclear morphology as an internal clock to confirm that observed changes in cytoplasmic morphology were indeed sequential.

Of the four enzymes studied, most information was gained from peroxidase. When HL-60 cells were induced to differentiate to neutrophils with agents such as DMSO, there was a progressive loss of peroxidase activity over the induction period. Koeffler et al[23] has shown this to be due to a cessation of synthesis of myeloperoxidase. On the other hand, when HL-60 cells were induced to differentiate to eosinophils, total peroxidase activity increased above baseline after an initial dip seen at 24 to 48 hours of induction. The appearance of a new spectral peak at 452 nm on the oxidized minus reduced difference spectrum of the extracts of HL-60 eosinophils demonstrated the presence of eosinophil peroxidase which was not present at the beginning of the induction. Because the peak at 474 nm is still seen at the end of the induction period, myeloperoxidase appears to be present at this point as well. Unfortunately, this approach does not lend itself to precise quantitation of the relative amounts of the two peroxidases. Whether the initial drop in total peroxidase activity represents some loss of myeloperoxidase with the latter increase in total activity representing new synthesis of eosinophil peroxidase must await future studies using alternative assay techniques. Nevertheless, this demonstrates qualitative, quantitative, and kinetic differences in the expression of peroxidase activity and its isoenzymes between HL-60 cells induced to differentiate to eosinophils and those induced to differentiate to neutrophils.

Cells at all stages of eosinophilic differentiation could be identified in electron photomicrographs of HL-60 eosinophils stained for peroxidase activity. The least morphologically mature cells, which clearly appeared to be the precursors for the more mature HL-60 eosinophils, stained for peroxidase activity only in the endoplasmic reticulum and the perinuclear cistern. Judging by the absence of cytoplasmic granules and the appearance of the nucleus, these cells corresponded most closely to myeloblasts. Although these immature cells demonstrated complete loss of the peroxidase-positive promyelocytic granules seen in the undifferentiated HL-60 cells, it is likely that the majority of HL-60 cells differentiating to eosinophils retain a portion of their promyelocytic granules. Our previous studies support this conclusion because toluidine blue staining in HL-60 eosinophils reveals the continued presence of promyelocytic granules in HL-60 cells with many eosinophilic granules by virtue of the former's metachromatic staining properties.[9] Furthermore, the observed decrease of peroxidase activity seen between 24 and 48 hours of induction (Fig 1) combined with the demonstration of myeloperoxidase activity (specific for promyelocytic granules) in the populations of HL-60 cells that have differentiated to eosinophils is consistent with partial loss of promyelocytic granules being typical.

As the cells continued to mature, further collection of flocculent peroxidase-positive material caused dilatation of the endoplasmic reticulum. Segments of the endoplasmic reticulum formed vacuoles enclosing this material. It then apparently condensed into spherical granules that remained peroxidase-positive. In some cases, a multilammelar crystallization of granule proteins formed that remained peroxidase-positive throughout the structure. These structures have been suggested by Dixon et al[21] to represent precursors of Auer rod formation in granules of acute promyelocytic leukemia cells. However, until typical, fully formed Auer rods are observed in HL-60 cells, firm identification of these structures as Auer rod precursors remains speculative.

In most sections of cells at all stages of maturation where Golgi apparatus can be visualized, little enzyme activity is seen within the structure. This observation must be interpreted with caution because microscopic observations can only demonstrate the presence of enzyme activity in a particular compartment, and in the absence of other data, cannot be used to draw kinetic or quantitative conclusions about the rates of passage of proteins through that compartment.[24] However, as sections of multiple cells have been taken, simultaneously showing abundant enzyme activity in the endoplasmic reticulum, vacuoles, and granules, with little in the Golgi apparatus, we conclude that the Golgi apparatus is not a major conduit of peroxidase from its point of synthesis to the granule. This conclusion is in agreement with Bainton et al[5] and Breton-Gorius and Houssay[6] who demonstrated decreased peroxidase staining of the Golgi apparatus in leukemic myeloid cells. Failure to observe formation of peroxidase-containing vacuoles budding from the Golgi apparatus is further evidence of lack of involvement of the Golgi apparatus in processing peroxidase before its transport to the vacuoles.

Scott and Horn have described a similar mechanism of granule formation.[25] In their study of normal eosinophil precursors, the endoplasmic reticulum segmentally dilated forming vacuoles that were partially filled with an electron dense matrix. They too noted little evidence of the Golgi apparatus packaging granule contents. However, neither they nor we unequivocally demonstrated a connection between the vacuoles and the endoplasmic reticulum once the granules began to form. Furthermore, it is impossible to exclude the possibility that both mechanisms (dilatation of segments of the endoplasmic reticulum containing preformed peroxidase, and transport of proteins through the Golgi apparatus) are simultaneously operative to variable extents. The recent demonstration that monensin can inhibit the processing of myeloperoxidase in normal eosinophils, but not that of eosinophil peroxidase, further supports the possibility of two pathways of processing of granule proteins.[26]

Acid phosphatase activity was not visualized in the endoplasmic reticulum or Golgi apparatus of the immature HL-60 eosinophils with either β-glycerophosphate (which stains only lysosomal sites) or p-nitrophenyl phosphate (which stains extralysosomal sites as well).[12] Deposits corresponding to enzyme activity were demonstrated, however, in the Golgi apparatus of other cells which appeared to be differentiating into neutrophils. Thus, the early events in acid phosphatase processing could not be observed in the eosinophils. However, as with peroxidase, the acid phosphatase activity appeared to be included in the flocculent material which condensed to form granules within the vacuoles. Unlike the peroxidase activity, the acid phosphatase activity appeared to be inactivated as it underwent condensation into granules. This type of enzymatic inactivation has been described in normal eosinophils as well.[22] In the granules that underwent crystallization to form multilamellar structures, the acid phosphatase activity appeared to have been inactivated in the areas where the crystallization was well developed but appeared to remain intact where the crystallization was not yet fully formed.

Eosinophilic differentiation of the HL-60 cells was associated with an increase in the total amount of acid phosphatase enzyme activity. Furthermore, the isoenzymes of the acid phosphatase expressed appeared to be similar to those seen in the mature HL-60 neutrophils. Thus, none of the isoenzymes appeared unique for either neutrophilic or eosinophilic differentiation of HL-60 cells.

Arylsulfatase activity and lysophospholipase activity were both detected in the mature HL-60 cells, but at very low levels. In our earlier studies, the presence of lysophospholipase was demonstrated by immunofluorescence techniques with an antiserum prepared against the Charcot-Leyden Crystal protein (identical to lysophospholipase).[9,17] Fluorescence was shown in only a small proportion of cells (maximally 6%), even after induction of differentiation. Therefore, expression of this particular protein is not a constant feature of eosinophilic differentiation of the HL-60 cells, Similarly, arylsulfatase activity was only seen as small deposits on the outside of granules, involving only a minor subpopulation of the cells. Thus, both of these enzymes, which are characteristic of normal eosinophils, appear to be expressed to considerably lower extents in the HL-60 eosinophils.

These data demonstrate several important differences between the HL-60 eosinophils and normal eosinophils. First, both myeloperoxidase and eosinophil peroxidase appear to be coexist in the population of mature HL-60 eosinophils, whereas normal eosinophils have little or no myeloperoxidase. Second, whereas the majority of authors who have examined the role of the Golgi apparatus believe it to be of major importance in the packaging of granule proteins in normal eosinophils, we observed only a variable, minor amount of enzyme activity present in the Golgi apparatus at any time. Third, low levels of arylsulfatase and lysophospholipase activity were demonstrated in the HL-60 cells compared to normal eosinophils and those from patients with secondary eosinophilia. Fourth, in some cells, it was possible to demonstrate structures that have been described as being precursors of Auer rods, whereas these are never seen in normal eosinophils. Taken together, these data suggest that there exists one or more disorders of granule protein synthesis and processing in the HL-60 eosinophils as compared to normal eosinophils. Because of the morphologic and histochemical similarity of the HL-60 eosinophils to those seen in patients with various malignant diseases, especially ANLL-M4eo, we conclude that these disorders of synthesis and processing are very likely responsible, at least in part, for the granular abnormalities seen in the eosinophils of those patients.

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Long-Term Culture of Bone Marrow-Derived Preleukemic Cells From F-MuLV-Infected Mice

By Jean Michel Heard, Brigitte Sola, Marie-Antoinette Martial, Serge Fichelson, and Sylvie Gisselbrecht

The replication-competent Friend leukemia virus (F-MuLV) induces leukemias involving three hematopoietic lineages after a latent period of several months. In an attempt to elucidate the early events of the leukemogenic process, we looked for a method allowing the isolation and the long term in vitro maintenance of preleukemic cells. When established as long-term cultures according to the technique described by Dexter et al,[1] bone marrow cells obtained from 7/7 apparently healthy F-MuLV-infected preleukemic mice led to the accumulation of immature myeloblastic cells, and to the generation of permanent myeloblastic cell lines, which in most cases further became tumorigenic in preirradiated recipient animals. The delays required to obtain cell lines were shorter when the duration of the in vivo infection was longer, suggesting that these cells were committed into the leukemogenic pathway before their transfer into culture flasks. The myelomonocytic preleukemic cells exhibited normal sensitivity to purified preparations of CSFs, but acquired the capacity to grow in the absence of exogenous CSF stimulation. Examination of integrated provirus copies demonstrated that the preleukemic cell proliferation involved a single or a few clones which may progress in vitro from a preleukemic to a fully malignant stage without major modifications of the integrated provirus copies.

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REPLICATION-competent murine leukemia viruses (MuLVs) are retroviruses devoid of oncogenes, that induce leukemias in infected mice after a delay of several months. It is obvious that the crucial events leading to the generation of leukemic cells take place during the latent preleukemic period. In most cases, preleukemic animals are clinically healthy, although anemia and splenomegaly are sometimes observed. A prolonged latent period and the existence of preleukemic myeloproliferative diseases have also been recognized in some human leukemias. This suggests that MuLV-induced long latency leukemias may be useful experimental models for human diseases. However, the understanding of the mechanisms by which MuLV infection leads to leukemias largely depends on the availability of an experimental tool allowing investigations on preleukemic cells.

MuLV-infected preleukemic lymphoid cells were initially detected in mice by Haran-Ghera.[2,3] They were recognized by their ability to induce leukemias of donor origin several months following transplantation into syngeneic recipients. However, all attempts to maintain lymphoid preleukemic cells in cultures have failed in the past.[4] Recently, O'Donnel et al[5,6] isolated preleukemic cells corresponding to successive stages in the development of AKR thymic lymphomas. Attempts to reproduce the preleukemic events were also performed by using the long term culture of murine bone marrow cells described by Dexter et al.[1] Thus, in vitro infection of normal bone marrow cells allowed to follow successive steps in the appearance of Friend virus (FVP)-induced erythroleukemia,[7] and led us to define successive stages in the myelomonocytic leukemic transformation induced by the helper component of FVP, the F-MuLV.[8,9]

F-MuLV-infected mice develop erythroid, lymphoid, and myelomonocytic leukemias after a latency of several months.[10-12] In most mouse strains, the latent period is silent; however, some susceptible strains show a severe anemia with hepatosplenomegaly which is probably not leukemic, and which regresses after blood transfusions.[13] In this paper, we describe the isolation, long-term culture, and in vitro progression toward malignancy of preleukemic cells issued from apparently healthy mice infected as young adults with the I^{-5} strain of F-MuLV.[14] We took advantage of the prevalence of the myelomonocytic progenitor growth and differentiation in the Dexter culture technique[1] to apply it to the long-term culture of the preleukemic myelomonocytic cells supposed to be present within the bone marrow cells of some F-MuLV-infected mice. Indeed, these cultures were progressively overgrown by immature myelomonocytic cells which gave rise to permanent cell lines. This allowed us to isolate preleukemic cells issued from infected animals and to examine their growth factor dependence and the pattern of integrated provirus copies.

MATERIAL AND METHODS

Virus and virus infection. The origin of the I^{-5} stock of F-MuLV has been described previously.[14] Batch used contained 2 × 10^6 focus-inducing units (FIU) per mL in the S^+L^- assay.[15]

Young adult female mice (6 to 8 weeks old) were infected by intraperitoneal injection of 10^5 FIU in a volume of 0.5 mL. For in vitro infection of bone marrow cells, 10^5 FIU in a volume of 1 mL were incubated for 1 hour at 37 °C on monolayer cells. 9 mL of 10% fetal calf serum (FCS) supplemented culture medium were further added into each culture flask.

Cell cultures. Long-term bone marrow cell cultures were established according to the technique reported by Dexter et al,[1] with a few modifications already described.[8,9] Uninfected bone marrow cells were first allowed to grow as an adherent cell layer for 3 weeks. Then, the content of a single femur from either a F-MuLV-infected mouse, or from a normal mouse, was added to the culture. When normal bone marrow cells were infected in vitro, F-MuLV was added simultaneously as described.[7,8] This date was considered as day zero for all the cultures. Cultures were refed twice a week. Once a week, cells collected with the supernatant were used for morphologic examination, for colony assays, and for attempts to establish secondary cultures and cell lines. In the latter case, one half of the culture medium was transferred into a new culture flask at successive intervals of time leading progressively to the elimination of the

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adherent cell layer. The same procedure was used to establish cell lines from leukemic animals, except that tumor cells were plated at a concentration of 2 to 5 × 10^5 cells per mL. Established cell lines were maintained as previously described.[8,9]

Cloning assays in agar cultures were performed as described in a previous paper.[16] Purified CSF-1,[17] GM-CSF,[18] and IL-3[19] were kindly provided by Dr Stanley, Dr Burgess, and Dr Ihle, respectively.

Mice, tumor transplantations, and tumorigenicity assays. (C57Bl/6 × BALB/c)F1 mice came from our own breeding and were maintained in pathogen-free conditions. Transplantations of tumors and tumorigenicity assays were performed in 450 rads preirradiated (^{60}Co source) isogenic male adult recipients. Cell-injected animals were regularly observed for the presence of any tumor until 6 months postinoculation. Tumors removed from moribund mice were used for cytologic studies, karyotypic analysis, and tranplantation into secondary recipients. The technique used for karyotypes has been described elsewhere.[8,9]

Detection of integrated F-MuLV genomes. High molecular weight DNA extraction and Southern blots[20] were performed exactly as previously described.[21] Briefly, 10 μg of EcoR1 digested DNAs were run on 0.8% horizontal agarose gels, transferred to nitrocellulose filters, and hybridized in stringent conditions with ^{32}P-labelled nick-translated probe (specific activity, 2 × 10^8 cpm/μg of DNA). Hind III digested lambda DNA was used as size marker.

RESULTS

(C57Bl/6 × BALB/c)F1 hybrids (B6C) have been used since (1) they bear the Fhe allele that confers resistance to the early erythroid disease[22]; (2) they are known to exhibit a long latent period and a high percentage of myelomonocytic leukemias after infection with the I^{-5} isolate of F-MuLV[23]; (3) their bone marrow cells are suitable for the Dexter's culture technique and the in vitro generation of myelomonocytic leukemic cells.[8,9]

Leukemia induction in F-MuLV–infected B6C mice. Twelve young adult B6C mice were infected with the I^{-5} strain of F-MuLV. As expected, none of them exhibited the early erythroid disease. In contrast, 11/12 developed leukemias after a latent period of 45 to 55 weeks, confirming the nearly 100% incidence of leukemias in F-MuLV–infected mice.[23] Determination of the cell lineage origin of the leukemic cells also confirmed the prevalence of the myelomonocytic leukemias (6/11). Bone marrow cells from mice developing myelomonocytic leukemias were used to establish permanent leukemic cell lines in order to compare their growth properties with those of the bone marrow cells issued from apparently healthy preleukemic mice. Figure 1 shows that cells with myeloblastic characteristics (excentred nucleus and myeloperoxidase positive cytoplasmic granules) rapidly grew. Permanent myelomonocytic cell lines were obtained within a few weeks, and were tumorigenic when injected into 450 rads preirradiated syngeneic mice. Tumors exhibited the cytologic characteristics of myeloblastic leukemias.

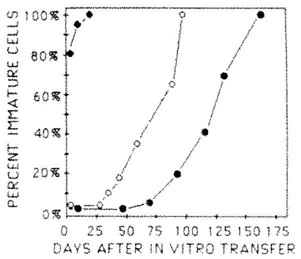

Fig 1. Time course of the percentage of immature cells in newly established leukemic and preleukemic bone marrow cell cultures. ♦: bone marrow cells from an overt F-MuLV–induced myelomonocytic leukemia; ● and ♦: cultures C and P established from bone marrow cells of mice 1 and 5, respectively, 17 and 40 weeks after the F-MuLV infection.

Establishment of myelomonocytic cell lines from preleukemic animals. Seven B6C mice were infected with the I^{-5} F-MuLV and killed during the latent preleukemic period. One mouse was killed at week 17 postinfection, and two mice were killed at weeks 27, 40, and 50, respectively. These animals were carefully examined to check up on the absence of an overt leukemia. Such control was critical for the animals killed at weeks 40 and 50, since the occurrence of leukemias was expected at this time. Mice were considered as healthy when spleen weight and cytologic examination of blood, spleen, lymph nodes, and bone marrow were normal. We also controlled that bone marrow cells formed colonies of fully mature granulocytes and macrophages when plated in agar cultures in the presence of colony stimulating factor (CSF). Bone marrow cells from each mouse were plated in three tissue culture flasks where a feeder layer of normal bone marrow cells had been previously grown (see Materials and Methods) (Fig 2). In the same time, three control uninfected cultures were established from normal B6C mice. Investigations on both preleukemic and normal mouse cultures included twice-a-week examination of the morphology of the nonadherent cell population and attempts to derive secondary cultures in the absence of any exogenous growth stimulating factor.

12/21 preleukemic mouse cultures evolved like uninfected controls. They contained 90% mature granulocytic and macrophagic cells, they were unable to generate secondary cultures, and they ended after 20 weeks (Fig 2).

In contrast, in 9 cultures, after a delay that varied

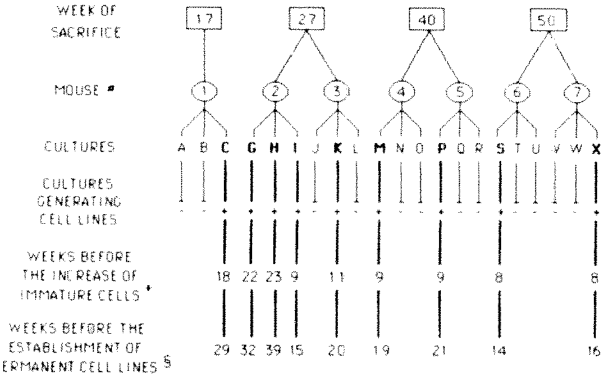
WEEK OF SACRIFICE
17
27
40
50
MOUSE #
1
2
3
4
5
6
7
CULTURES
A B C G H I J K L M N O P Q R S T U V W X
CULTURES GENERATING CELL LINES
WEEKS BEFORE THE INCREASE OF IMMATURE CELLS *
18 22 23 9 11 9 9 8 8
WEEKS BEFORE THE ESTABLISHMENT OF PERMANENT CELL LINES §
29 32 39 15 20 19 21 14 16

Fig 2. Experimental schedule and delays needed to isolate preleukemic cells. Mice were picked up randomly within a cage of 20 infected B6C mice from which obviously leukemic animals had been removed. Cultures were established using either 1 femur or 2 tibias. Cultures that did not generate cell line ended between 20 and 30 weeks. *date when the percentage of immature cells reached 50%. §date of the first successful transfer without feeder-adherent cell layer.

according to the culture, the initial fully maturing cell population was substituted by immature cells with excentred nucleus and myeloperoxidase positive cytoplasmic granules. The percentage of immature cells progressively raised 100% (Fig 1). All these cultures were able to generate secondary cultures and later establish cell lines simply by successive transfers of the nonadherent cells into new culture flasks (Fig 2). Neither feeder cell layer nor addition of exogenous growth factor were required. We examined each month the capacity of the cells from primary cultures, secondary cultures, and established cell lines to induce tumors of donor origin when injected into 450 rads preirradiated syngeneic recipient mice. Tumor induction was never observed when cells were inoculated before they could be established as permanent cell line. 6/9 cell lines became tumorigenic. They induced either generalized leukemias or subcutaneous tumors within the inoculation area, or both. The donor origin of tumor cells was controlled by karyotypic analysis, female cell lines being injected into male recipients. Delays for the first engraftment varied from 6 to 12 weeks according to the cell line. Tumors always exhibited the cytologic characteristics of myeloblastic cells. They were transplantable into preirradiated mice and easily adapted to in vitro growth.

Figure 2 shows that 9 cell lines were obtained from 7 F-MuLV–infected mice, and that each sacrificed animal gave rise at least to one permanent cell line. Since no cell line could be derived from uninfected animals, we concluded that the capacity of bone marrow cells to generate permanent cell lines resulted from the F-MuLV infection despite the absence of any clinical expression of the viral infection.

Myelomonocytic cell lines are derived from preleukemic cells present in F-MuLV–infected mice. We have previously shown[8,9] that the in vitro F-MuLV infection of normal bone marrow cell cultures leads to the generation of malignant myeloblastic cell lines in a very similar way to that observed here with preleukemic mouse cultures. Therefore, the question arose of whether the cells originated from infected mice underwent the entire leukemic process after their transfer in vitro, or whether they were already committed into the leukemic pathway at the time of seeding into culture flasks, and could actually be considered as preleukemic cells.

To examine this point, we compared the delays preceding both the increase in the percentage of immature cells and the establishment of permanent cell lines when bone marrow cells were either obtained from infected mice (17, 27, 40, and 50 weeks after the infection), or obtained from uninfected mice and further infected after their in vitro transfer. For this purpose, 8 bone marrow cultures established from normal mice were infected 3 weeks later with the I^{-5} F-MuLV strain. After a period during which infected cultures resembled uninfected controls (12 to 32 weeks according to the culture), the cell production progressively switched toward the generation of immature cells in 5/8 cultures, and cell lines were established a few weeks later.

Figure 3 shows the delays preceding the increase in immature cells (Fig 3A) and the establishment of cell lines (Fig 3B) after both in vivo (black circles) and in vitro (white circles) F-MuLv infection. Data are expressed in terms of the duration of the in vivo infection (this value being zero for in vitro infected cultures, and 17, 27, 40, and 50 for cultures derived from infected animals.) Calculation of the correlation index between delays and duration of the in vivo infection reveals a significant inverse relationship for both the increase of the immature cells (Fig 3A, r = −0.64, $P < 0.02$) and the establishment of permanent cell lines (Fig 3B, r = −0.70, $P < 0.01$). Acceleration of in vivo versus in vitro infected cultures was not due to different levels of virus production since titers of replication-competent MuLVs, as measured by foci induction in S^+L^- cell cultures,[15] were similar in both cases (data not shown). Furthermore, shortening of delays also appeared within the group of the in vivo infected cultures (Fig 1 and Fig 2). A longer period of in vivo infection was related to shorter delays before the increase of immature cells and the establishment of permanent cell lines. These data suggest that in F-MuLV–infected mice some hematopoietic cells had already entered the leukemic pathway at the time of seeding in culture, and thus could proceed toward malignancy more rapidly than normal bone marrow cells infected in vitro.

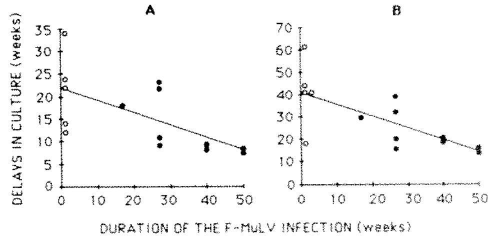

Fig 3. Relationship between the duration of the in vivo F-MuLV infection and the delays needed to isolate preleukemic cells from bone marrow cultures. (A) Delays before the increase of the percentage of immature cells. Plotted points correspond to the date when the percentage of immature cells reached to 50%. (B) Delays before the establishment of permanent cell lines. Plotted points correspond to the date of the first successful transfer without feeder-adherent cell layer. ○: data from bone marrow cell cultures infected in vitro. Duration of the in vivo infection is zero; ●: data from bone marrow cell cultures established from infected mice. Duration of the in vivo infection is 17, 27, 40, or 50 weeks according to the culture. Correlation index had been calculated from each set of individual points according to the formula:

$$r = \frac{\Sigma(x_i - mx)(y_i - my)}{\sqrt{\Sigma(x_i - mx)^2 \Sigma(y_i - my)^2}}$$

where x_i are the duration of the infection in mice, y_i are the delays preceding either the increase of the immature cells or the establishment of permanent cell lines, and mx and my are the algebraic averages of x_i and y_i, respectively. Regression curves were calculated according to the equation $y = my - [(r)(mx)]$.

CSF dependence of early preleukemic myelomonocytic cells. Establishment of permanent cell lines from preleukemic mouse bone marrow cultures was always preceded by two periods: (1) an initial period during which the cultures produced fully maturing cells exactly as did control cultures, (2) an intermediate period characterized by the overgrowth of immature cells while permanent suspension cell lines could not be yet established. We already described such a time course in cultures infected in vitro,[9] and we showed that the intermediate period was characterized by a transient dependence of the cells toward the exogenous stimulation by CSFs.[9]

We examined whether this was also the case in preleukemic cell cultures. For this purpose, nonadherent cells from the culture C were harvested as soon as the percentage of immature cells raised 50%, and were plated in agar cultures in the presence of increasing concentrations of crude and purified preparations of CSFs. Dose-response curves allowed us to estimate the sensitivity of the cells to the various growth-stimulating factors, and to compare it with the one of normal bone marrow cells plated in the same time. Results are shown in Fig 4. No colony was obtained in the absence of stimulating factor. Colony formation was induced by the addition of WEHI-3B–conditioned medium (CM) which is known to contain CSF,[24] and by purified CSF-1,[17] GM-CSF,[18] and IL-3.[19] Sensitivities of the preleukemic cells to CSFs were either similar or lower than those of the normal bone marrow cells. Colonies induced in the presence of CSFs only contained immature myelomonocytic cells. Cells picked up from WEHI-3B CM–induced colonies generated continuous suspension cell lines when transferred in liquid cultures in the presence of the same stimulating factor. Cell morphology remained unchanged even after several weeks of culture in the presence of CSF-containing medium. However, the dependence toward exogenous stimulating factor was lost after 10 weeks. At this time, factor-independent cell lines could be established from the original bone marrow culture, and colony formation could be observed in the absence of added exogenous stimulating factor (data not shown).

We concluded that during a transient period, the early preleukemic myelomonocytic cells remain dependent toward their physiologic growth-stimulating factor (GSF) despite their inability to express the granulomacrophagic differentiation program as do their normal counterparts.

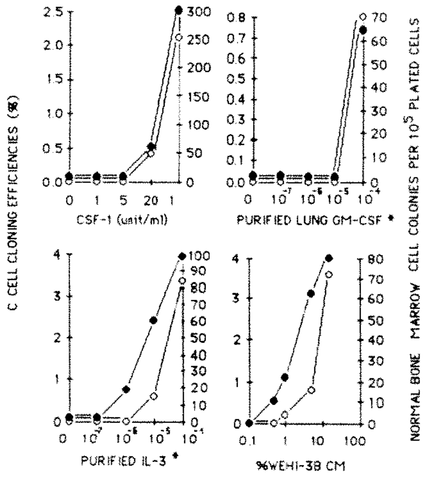

Fig 4. Stimulation of preleukemic cell colony formation by various sources of CSF. ◊—◊: percentage of colony-forming cells (cloning efficiency) in preleukemic cells removed from the culture C. Cells were plated at a concentration of 2.5 × 10³ cells per mL; ♦—♦: colony formation of normal bone marrow cells plated at a concentration of 5 × 10⁵ cells per mL. *Concentrations of purified GM-CSF and purified IL-3 are given in term of dilutions of the original batches provided by Dr Burgess and Dr Ihle. Each determination was done in duplicate.

Integrated F-MuLV provirus copies in cultured myelomonocytic preleukemic cells. Clonality of the proliferating tumor cells has been previously demonstrated in murine long latency virus-induced neoplasias.[25,26] The isolation of myelomonocytic preleukemic cells from F-MuLV–infected mice allowed us to examine the cell clonality at the early stages of the leukemic process. For this purpose, we looked at the integrated provirus copies by using an ecotropic virus-specific DNA probe (E57BS) subcloned from the F-MuLV genome.[27] We already used such a procedure to demonstrate that the in vitro leukemic transformation induced by the F-MuLV resulted from a clonal expansion.[21] By Southern blot analysis, the E57BS probe detects a single 3.5 Kb band in the EcoR1 digested DNA of noninfected mouse cells (Fig 5A, lane 1). Analysis of DNA from F-MuLV–induced myelomonocytic leukemic cells showed a limited number of integrated provirus copies (Fig 5A, lane 2) indicating a clonal origin of these cells. DNAs were extracted from the nonadherent cells of bone marrow cultures established from preleukemic F-MuLV–infected mice. When examined before the onset of the immature myelomonocytic cells the E57BS probe detected a smear (Fig 5B, lane 1), showing a random integration of F-MuLV proviruses in these cells, and therefore the polyclonality of the cell population. In contrast, as early as the cultures were overgrown by immature myelomonocytic cells, and before any subcloning of the cell population, discrete numbers of band were detected (Fig 5B, lane 2). Grossly the same hybridization pattern was conserved all along the in vitro proceeding of the cells, and also in the various tumors induced by their in vivo engraftment with the exception that some additional bands and/or deletions have been detected in some cases (Fig 5C, lanes 1, 2, 3). This provided evidence that a small number, or more probably a single preleukemic clone, progressively emerged from the infected cell population sometimes before the increase of the immature cell percentage could be detected.

DISCUSSION

The replication-competent Friend leukemia virus (F-MuLV) is characterized by a multiple hematopoietic tropism involving the lymphoid, the erythroblastic, and the myelomonocytic cell lineages.[10-12] In this paper we show that although apparently healthy, preleukemic F-MuLV–infected B6C mice possess myelomonocytic cells already committed into the leukemic pathway. These cells have been recognized by their capacity to overgrow bone marrow cultures established from preleukemic animals. They consist in a monomorph population of immature cells with myelomonocytic characteristics. They generate permanent autonomous cell lines growing in suspension, and in most cases, become tumorigenic as evidenced by tumor formation in preirradiated recipient mice. Our results also show that the preleukemic cell proliferation is monoclonal, and that the same clone proceeds in vitro from the preleukemic stage until the full malignancy.

In a previous work, we showed that the in vitro F-MuLV infection of normal hematopoietic cells generated malignant myelomonocytic cells.[8,9] Consequently, it might now be objected that the cell lines we derived from F-MuLV–

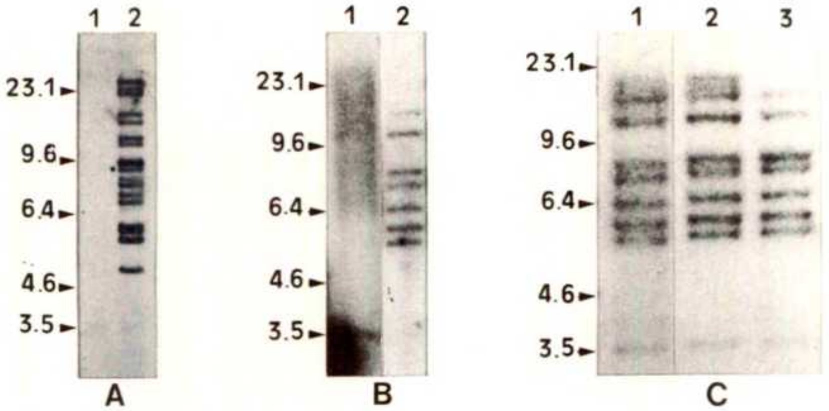

Fig 5. Analysis of integrated F-MuLV provirus copies in preleukemic bone marrow cell cultures. Southern blots of EcoR1-digested DNAs were hybridized with the nick-translated E57BS probe. Panel A: uninfected B6C mouse bone marrow cells (lane 1), cell line established from an overt myelomonocytic leukemia (lane 2); Panel B: P bone marrow culture established from mouse No. 5 killed 40 weeks after F-MuLV infection. Analysis of cells removed before the onset of the immature cells (less than 5% immature cells) (lane 1), and when the percentage of immature cells reached 50% (lane 2). Panel C: cells from the P cell line analyzed when established as a permanent suspension cell line (lane 1), and as tumor cells from two different recipient animals (lanes 2 and 3).

infected mice resulted from hematopoietic progenitors merely infected rather than from truly preleukemic cells. Since preleukemic cells were supposed to need shorter time to reach the leukemic stage than cells infected de novo within the culture flask, we compared the delays preceding transformation when either bone marrow cells infected in vitro or cells from preleukemic mice were used to establish the cultures. We observed that the overgrowth of the cultures by the immature cell population and the generation of permanent cell lines occurred more rapidly when the bone marrow cells came from preleukemic mice. This acceleration was related to the duration of the in vivo infection. It was very unlikely that such an acceleration might result from a higher susceptibility to in vitro virus transformation of old mouse bone marrow cells. Indeed the in vivo susceptibility to virus infection is known to decrease in old animals[29] and if considered in terms of frequency of transformation, the susceptibility of the cultures obtained from old mice was not increased. Furthermore, since the quality of the cultures established from old mice was rather poor, and since high quality cultures are required for in vitro transformation, it is very unlikely that the use of old bone marrow cells improved the efficiency of this leukemogenic process. Therefore we suppose that the shorter delays observed with cells issued from preleukemic mice resulted from their previous commitment into the leukemic pathway.

Surprisingly, we obtained myelomonocytic preleukemic cell cultures from the 7/7 mice that have been killed during the latent preleukemic period. This contrasts with the incidence of the myelomonocytic leukemias in the B6C strain which affects only 50% of the F-MuLV–infected animals. It suggests that myelomonocytic preleukemic cells were present in animals which would later have developed erythroblastic or lymphoid leukemias. This result fits with the known multiple tropism of F-MuLV. It indicates that the initiation of the leukemic process involves several hematopoietic cell lineages within the same animal, and it is consistent with the hypothesis that the F-MuLV target cell would be either the multipotential stem cell or a committed stem cell of each of the various lineages.

We observed that preleukemic cells transiently required a stimulation by exogenous sources of CSFs to form colonies in semisolid agar cultures and to proliferate at low cell density. We previously reported that, in Dexter's bone marrow cultures, CSFs are secreted by the adherent cell layer.[30] It may be assumed that this secretion provides a stimulation that enables the survival and proliferation of the myelomonocytic preleukemic cells, while preleukemic cells from the other hematopoietic lineages are lost in these particular culture conditions. However, although the stimulation by CSFs seems crucial for the isolation of preleukemic myelomonocytic cells, it is probably not responsible for the overgrowth of the cultures by the immature population. Indeed, as shown in Fig 4, preleukemic cells exhibited a sensitivity to CSFs similar to the one observed for uninfected bone marrow cells. This contrasts with data obtained in the erythroid lineage where hyperesponsiveness to traces of erythropoietin had been observed in human polycythemia vera[31] and in murine erythroleukemias induced by the anemia-inducing strain of the Friend virus.[32] We showed elsewhere[16] that during the in vitro myelomonocytic transformation induced by F-MuLV, the same level of sensitivity to CSFs was conserved along the leukemic pathway, and that the existence of an autocrine stimulation of growth did not account for the autonomous cell proliferation.

The monoclonal origin of long latency neoplasias has been previously reported.[25,26] Our analysis of integrated ecotropic provirus copies in the genome of preleukemic and leukemic cells provides evidence that in F-MuLV–infected mice, a monoclonal or oligoclonal population of transformed cells already exists during the latent period. These results are in agreement with recent data from O'Donnell et al[6] who reported the presence of clonal cell populations at the preleukemic stages of the AKR leukemias.

The concept of a preleukemic cell is rather formal and its definition varies according to the authors. Haran-Ghera et al using cells obtained very early after the virus infection, retained as a criterion the development of tumors of donor origin in recipient animals, while delays were similar to those of the virus-induced diseases.[2,3] O'Donnell et al[5] reported that a subpopulation of thymocytes isolated from preleukemic AKR mice 40 to 50 days after MCF virus infection induced accelerated tumor formation in preirradiated recipients. Oliff et al obtained permanent factor-dependent cell lines from mice exhibiting the F-MuLV–induced early erythroid disease (which precedes the occurrence of the leukemias); however, these cells needed the further transfection of the *abl* oncogene to become tumorigenic.[33] No activation of the *abl* oncogene was detected in our cell lines (unpublished data). We obtained evidences that the cell populations we isolated from preleukemic mice were clonal, we showed that the same clone became later tumorigenic, and that in these cells, the progression toward malignancy was shorter than after in vivo or in vitro de novo infection. We assume that these are valuable criteria for the definition of a preleukemic stage, and that our cells may actually be considered preleukemic.

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Polymorphonuclear Leukocyte Heterogeneity in Neonates and Adults

By Peter J. Krause, Harry L. Malech, James Kristie, Catherine M. Kosciol, Victor C. Herson, Leonard Eisenfeld,
William T. Pastuszak, Alex Kraus, and Bruce Seligmann

We have used a mouse monoclonal antibody (31D8) to determine whether differences in neutrophil (PMN) subpopulations might help explain decreased PMN chemotaxis in neonates compared with that of adults. 31D8 has been shown to bind heterogeneously to adult PMNs. Approximately 80% of the PMNs that strongly bind 31D8 (31D8 "bright") are the same cells that depolarize and migrate chemotactically when stimulated with the chemoattractant N-formyl-methionylleucylphenylalanine, while the 20% that weakly bind 31D8 fail to similarly respond. All neonatal PMNs bound 31D8 heterogeneously. There was a smaller population of 31D8 "bright" cells in neonates at birth (76% ± 6%, n = 45) compared with that of neonates at three to 15 days of age (82% ± 5%, n = 10, $P < 0.002$) and both were smaller than that of adults (88% ± 4%, n = 45, $P < 0.001$ and $P < 0.001$). Neonatal cord PMNs, which traversed a micropore filter in a modified Boyden chemotaxis chamber in the presence of a chemoattractant, had an increased percentage of 31D8 "bright" cells (89% ± 7%) than did PMNs which remained above the filter (82% ± 7%, n = 10, $P = 0.034$). PMN chemotaxis was less in neonates at birth (32.7 ± 4.5 μm) than at three to six days of age (36.8 ± 11.3 μm) and both were decreased compared with that of adults (69.1 ± 12.4 μm, $P < 0.001$ and $P < 0.001$). These findings indicate that decreased PMN chemotaxis in neonates may be due in part to a smaller PMN subpopulation of highly motile cells.

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THE immature chemotactic function of polymorphonuclear leukocytes (PMNs) in newborn infants is thought to be an important cause of their increased morbidity and mortality due to infection.[1-5] The mechanisms responsible for decreased PMN chemotaxis are not clear although both cellular and humoral abnormalities have been identified.[1-5] Previous studies of PMN chemotaxis in neonates have assumed a homogeneous population of mature cells with heterogeneity of response attributed to less motile immature PMNs. It is clear, however, from studies in adults, that there is a heterogeneous chemotactic response among PMNs that appear mature by light microscopy with approximately 60% to 80% of cells being classified as fast movers.[6-13] It has been shown that one cause of impaired PMN chemotaxis in neonates is an increased percentage of immature PMNs compared with that of adults.[4] The possibility that decreased neonatal PMN chemotaxis might also be due to a smaller percentage of mature, fast moving PMNs in neonates has not been investigated.

Recently, an IgG mouse monoclonal antibody (31D8) has been developed that binds heterogeneously to adult PMNs from the peripheral circulation and to PMN precursors in the bone marrow as early as the myelocyte stage of maturation.[14,15] The antigenic heterogeneity demonstrated by 31D8 binding correlates with PMN functional heterogeneity. Approximately 80% of the PMNs that strongly bind 31D8 (31D8 "bright") are the same cells that depolarize, reduce nitroblue tetrazolium, and migrate chemotactically when stimulated with the chemoattractant N-formyl-methionylleucylphenylalanine (f-met-leu-phe), while the 20% that weakly bind 31D8 (31D8 "dull") fail to similarly respond.[14,15] We studied PMN 31D8 binding and PMN chemotaxis in neonates and adults and noted a significantly smaller percentage of 31D8 "bright" cells and decreased chemotaxis in neonates than in adults. These results suggest that chemotaxis and possibly other functional differences between neonatal and adult PMNs may be explained, at least in part, by differences in PMN subpopulations.

METHODS

Study population. Blood was obtained from the fetal side of placentas (neonatal cord blood) within five minutes of birth, and from healthy, term neonates three to 15 days old. In each case blood was simultaneously drawn from healthy adult volunteers on no medication. Placentas were obtained from healthy women delivered vaginally or by repeat caesarean section who had local anaesthesia, no complications of pregnancy, labor, and delivery, and whose babies were in good health. Blood from older neonates was obtained at the time of heel stick blood sampling for phenylketonuria, a test performed on all newborn infants. All blood samples were immediately mixed with preservative free sodium heparin (50 U/mL blood) and brought to the laboratory for analysis. Informed consent to sample blood was obtained in accordance with the Hartford Hospital Human Investigation Committee guidelines.

Preparation and 31D8 labeling of PMNs. Whole blood was purified using 6% Hetastarch (McGaw Laboratories, Irvine, Calif) that yielded a population of cells composed primarily of PMNs and monocytes as previously described.[16] In some cases the cell population was further purified by Ficoll-hypaque separation yielding a preparation of greater than 90% pure PMNs. Labeling of PMNs with 31D8 antibody was performed as described previously.[14,15] In brief, separated PMNs were washed twice in PBS-BSA, counted, mixed with 31D8 antibody, and allowed to incubate on ice for 45 minutes. The PMNs were then washed three times in PBS-BSA and incubated for 30 minutes on ice with fluorescein isothiocyanate labeled goat-antimouse IgG. The labeled PMNs were washed three more times in PBS-BSA and stored on ice, in the dark, and in the absence of Ca^{2+} or MG^{2+} until use. Separated and labeled cells remained alive as shown by trypan blue exclusion. The following terminology is used in the manuscript for each study group. "adult PMNs" refer to PMNs isolated from the venous blood of adults, "neonatal cord PMNs" refer to PMNs isolated from the cord blood of newborn infants, and "older neonatal PMNs" refer to PMNs isolated from the venous blood of three- to 15-day-old infants.

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Analysis by flow cytometry. Flow microfluoremetry was performed using an EPICS C (Coulter, Inc, Hialeah, Fla) with an argon laser emitting 500 mw at 488 n. Forward and 90° light scatter were used to identify the PMN population from lymphocytes and monocytes. The fluorescence intensity distribution of 10,000 PMNs was determined. The data was presented as the number of cells on the vertical axis and log green fluorescence on the horizontal axis divided into a three decade log scale consisting of 255 channels. In some experiments cells were sorted according to fluorescence intensity for further analysis. Gain settings for the photomultiplier tubes for 90° light scatter, forward light scatter, and green fluorescence were gain 5, PMT 400; gain 5; and gain 5, PMT 1300, respectively, and were constant for neonates and adults in all experiments.

PMN locomotory assay. PMN locomotory response was determined using transparent acrylic modified Boyden chambers (Ahlco Corp., Southington, Conn) and nitrocellulose filters (140 μm thick, 5 μm pore size, Nuclepore, Pleasanton, Calif). The assay procedures have been described in detail elsewhere.[16,17] Cells were incubated for one hour with either f-met-leu-phe 10^{-8}_{M} or 3% zymosan activated serum in Medium 199. The results of the nitrocellulose filter assay were assessed using the microsectioning technique which describes the average distance migrated by the cells (locomotion index or LI_{20}).[17] PMNs incubated with the polycarbonate filter were separately harvested from the upper and lower compartments of the Boyden chambers, labeled with 31D8, and analyzed on the flow cytometer.

Statistical analysis. Significance of differences between groups were evaluated using the Student's t test. A P value of less than 0.05 was considered significant.

RESULTS

31D8 binding to adult and neonatal PMNs. The 31D8 antibody bound to neonatal cord PMNs, older neonatal PMNs, and adult PMNs in a heterogeneous manner. The majority of PMNs strongly bound 31D8 (31D8 "bright") and generated a highly fluorescent peak. The remaining PMNs weakly bound 31D8 (31D8 "dull") and generated a contiguous, smaller, less fluorescent area to the left of the major peak. Three typical neonatal cord PMN and adult PMN curves are shown in Fig 1. The 31D8 bright peaks are similar. The 31D8 dull areas are more variable including a distinct peak (1A) or a "shoulder" to the left of the 31D8 bright peak that varies in size (1B–F). PMNs from adults are more concentrated in the bright areas than PMNs from neonates. The adult bright peak in 1D has only a very slight left shoulder and while the other adult curves (1E, F) have larger dull shoulders, they are smaller than those of most neonates. In order to quantitate the size of the bright and dull areas, it was assumed that the 31D8 bright population generated a symmetric curve and that any other cells were part of the dull population.[15] Using the flow cytometer, the area of the entire curve was determined and the area of the symmetric curve about the major peak (bright curve) subtracted from the total area to determine the size of the dull area.

Significant differences in 31D8 antibody binding to neonatal cord PMNs, older neonatal PMNs, and adult PMNs were noted with this analysis as shown in Table 1. Neonates at birth had a smaller population of 31D8 bright cells (76% ± 6%) than neonates three to 15 days of age (82 ± 5%, $P < 0.01$), and both had a smaller population of 31D8 bright cells compared with that of adults (88% ± 4%, $P < 0.001$, and $P < 0.001$). The mean skew and kurtosis of the curves

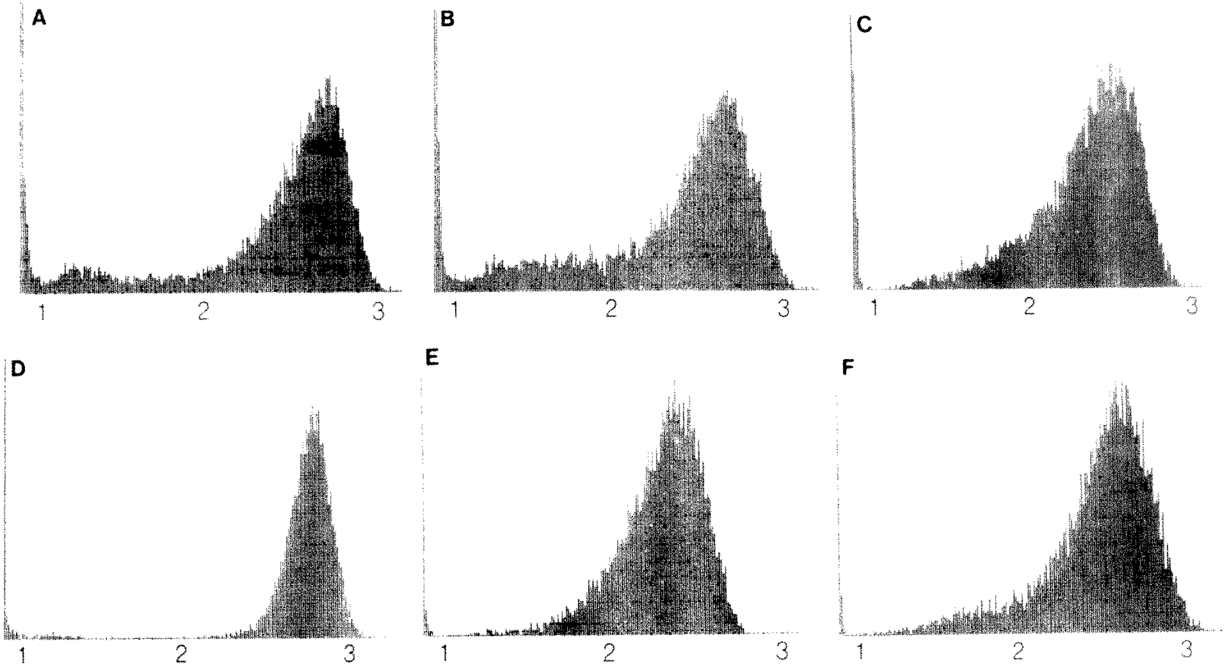

Fig 1. 31D8 antibody binds PMNs heterogeneously. The illustrations are representative curves of 31D8 antibody binding to PMNs in neonates (1A–C) and adults (1D–F). In 1A there is a major peak consisting of 31D8 bright PMNs and a minor peak of 31D8 dull PMNs. In 1D-F there is a single major peak of 31D8 bright PMNs with a "shoulder" or "tail" to the left of the major peak consisting of 31D8 dull PMNs. The horizontal axis depicts fluorescence intensity with cells to the left of 1 exhibiting autofluorescence, 1–2 one log fluorescence, and 2–3 two log fluorescence. The vertical axis depicts the number of PMNs. The percentage of 31D8 bright cells are as follows: 1A: 65%, 1B: 69%, 1C: 77%, 1D: 91%, 1E: 87%, 1F: 86%.

Table 1. 31D8 Monoclonal Antibody Binding to Adult and Neonatal PMNs

		Mean Fluorescent Antibody Distribution Characteristics		
Subjects	n	31D8 Bright Cells (%)	Skew	Kurtosis
Adults	45	88 ± 4 (a)	−1.8 ± 0.8 (d)	11.5 ± 4 (g)
Neonates (3 to 15 days old)	10	82 ± 5 (b)	−1.8 ± 0.5 (e)	7.2 ± 2 (h)
Neonates (Birth)	45	76 ± 6 (c)	−1.4 ± 0.5 (f)	5.8 ± 2 (i)

Significant differences ($P < 0.01$) were noted among the groups in each column except for (h) *v* (i), $P < 0.05$, and (d) *v* (e), $P = 0.8$.

were also determined as another method to compare neonatal and adult 31D8 binding. The neonatal fluorescence curves had a decreased mean skew and kurtosis compared with those of adults (Table 1). There was no significant difference in 31D8 bright populations in females or males in adults (89% ± 3%, and 88% ± 4%, respectively), neonates at birth (77% ± 5%, and 75% ± 6%, respectively), or neonates three to 15 days of age (84% ± 3% and 81% ± 5%, respectively).

Differences in 31D8 fluorescence intensity between adult PMNs, neonatal cord PMNs, and older neonatal PMNs were not due to cell size, as determined by comparing forward light scatter on the flow cytometer for the 31D8 bright and dull subpopulations in the three groups. There was also no difference in the location of antibody binding to neonatal cord PMNs, older neonatal PMNs, and adult PMNs. In all cases there was uniform distribution of fluorescently tagged 31D8 around the periphery of the PMNs. PMN activation with f-met-leu-phe for 60 minutes at 37 °C did not affect the bright/dull cell ratio. There was no difference in the percentage of bright cells in PMNs incubated in Medium 199 or f-met-leu-phe from four neonates at birth (79% ± 5% and 81% ± 6%, respectively) or from four adults (90% ± 3% and 89% ± 4%, respectively).

Because of the possibility that the differences observed in 31D8 binding between neonates and adults were due to an increased percentage of immature PMNs (bands and metamyelocytes) in neonates, we labeled PMNs from three neonates (cord blood) and three adults with 31D8, sorted them into 31D8 bright and dull subpopulations, and analyzed the subpopulations for percentage of mature and immature PMNs. There was no signficant difference in the percentage of immature PMNs in 31D8 bright and dull subpopulations for neonates (16% ± 3%, and 21% ± 11%, respectively), adults (13% ± 7% and 17% ± 11%, respectively), or the total group of neonates and adults (15% ± 5% and 19% ± 10%, respectively).

Correlation of 31D8 binding and PMN chemotaxis. Neonatal cord PMNs from four individuals were incubated in Boyden chambers, collected above and below polycarbonate filters after migration in response to 3% zymosan activated serum, and labeled with 31D8. PMNs that traversed the filter had a log greater mean fluorescence intensity than PMNs that remained above the filter (Fig 2). PMNs that traversed the filter also had a higher mean percentage of 31D8 bright cells (89% ± 7%) than PMNs that remained above the filter (82% ± 7%, $P = 0.034$). These observations indicate that a motile PMN subpopulation in neonates expresses a larger amount of 31D8 antigen on its surface than a less motile subpopulation of cells.

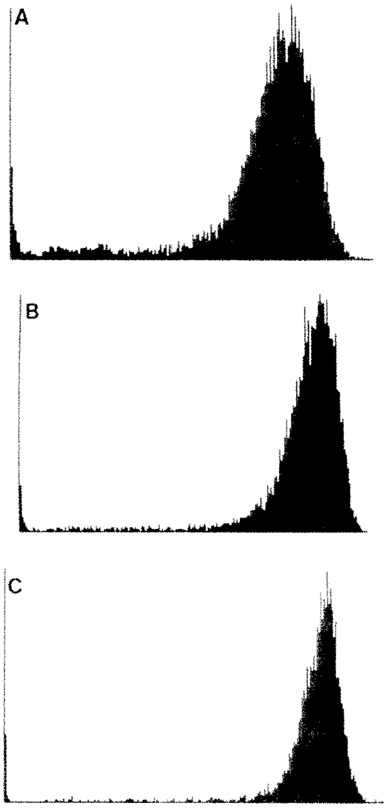

Fig 2. A motile subset of PMNs have a higher 31D8 fluorescence intensity than the total PMN population. In a representative experiment, PMN aliquots were labeled with 31D8 directly (A) and after one hour incubation in a Boyden chamber at 37 °C with 10_{M}^{-8} fmlp in the lower chamber (B, C). PMNs were collected from above the micropore filter (B) and below the micropore filter (C). The horizontal axis depicts fluorescence intensity using the same scale as in Fig 1. The vertical axis depicts the number of PMNs.

The difference in 31D8 expression in the "fast" PMNs that traversed the filter and "slow" PMNs that did not traverse the filter was not due to cell activation during passage through the filter since the percentage of bright cells was not affected by preincubation with f-met-leu-phe as mentioned previously. Furthermore, studies of Seligmann et al[15] showed that when PMNs are prelabeled with 31D8 and placed in the Boyden chamber, the population of cells that traverse the micropore filter into the bottom chamber have a higher percentage of 31D8 bright cells than those that remain in the upper compartment. This data shows that possible alteration in 31D8 expression by contact with the filter or exposure to greater concentrations of f-met-leu-phe does not account for differences in 31D8 expression between the "fast" and "slow" PMNs.

PMN chemotaxis using f-met-leu-phe and 31D8 antibody binding were simultaneously tested in five neonates at birth and adults. The mean chemotactic and 31D8 binding values for neonates were 28.8 ± 4.6 µm and 76.8% ± 6.2%, respectively, and for adults 52.7 ± 9.7 μm, ($P < 0.001$) and 88.8% ± 3.9%, $P < 0.001$), respectively. PMN chemotaxis using 3% zymosan activated normal serum was studied in six other neonates at birth and again at three to six days of age and in 12 healthy adults. Mean chemotactic (LI_{20}) values were 32.7 ± 4.5 μm, 36.8 ± 11.3 μm, and 69.1 ± 12.4 μm ($P < 0.001$ and $P < 0.001$, respectively). Thus, the magnitude of both the PMN chemotactic response and 31D8 antibody binding were smallest in neonates at birth, increased in neonates three to 15 days of age, and greatest in adults.

DISCUSSION

The results of this study demonstrate a clear difference in 31D8 monoclonal antibody binding to PMNs between neonates and adults. There was heterogeneous binding to PMNs of both groups, but neonates had a smaller percentage of cells that strongly bound 31D8 (31D8 bright) compared with that of adults. The percentage of 31D8 bright cells in neonates three to 15 days of age was intermediate between that of neonates at birth and adults. Several possible explanations for these differences were examined. The location of antibody binding sites on PMNs from neonates and adults was similar with uniform distribution of fluorescently tagged 31D8 antibody over the cell surface. There was also no difference in cell size of the 31D8 bright and dull subpopulations in neonates and adults as demonstrated by similar forward light scatter on the flow cytometer. There were no differences in eosinophil counts between neonates and adults. A higher eosinophil count in neonates could have accounted for the difference in 31D8 antibody binding since eosinophils weakly bind 31D8. Finally, although there was a greater percentage of immature PMNs (bands and metamyelocytes) in neonates than in adults, immature PMNs were equally distributed in the 31D8 bright and dull subpopulations. It is possible that 31D8 dull cells are less differentiated than 31D8 bright cells and that light microscopy is not sensitive enough to detect subtle PMN maturational changes.[6] This is unlikely, however, because 31D8 expression is not a late maturational event in PMN development but appears at the level of the myelocyte or metamyelocyte in the bone marrow.[15] Furthermore, a recent study by Gallin et al of 31D8 antibody binding to PMNs of patients with chronic myelogeneous leukemia suggests that 31D8 bright or dull cells may reflect true PMN subpopulations that originate from distinct stem cells.[18]

The cause for the difference in 31D8 binding in healthy neonates and adults is unclear. Since 31D8 binding is not affected by cell activation, changes in 31D8 binding must be due to increased production or destruction of 31D8 subpopulations. It is probably not due to differences in circulating estrogens or progesterone since unpublished observations by us of 31D8 PMN binding in seven pregnant women showed no difference in 31D8 binding during pregnancy and postpartum compared with nonpregnant control subjects. Neonates have a marked neutrophilia at birth that persists for 48 to 72 hours. Recent studies in neonatal rats indicate that the size, number of cell divisions, and rate of production of PMNs is different in neonates than in adults.[19,20] These differences might account for PMN 31D8 subpopulation differences between neonates and adults.

The PMN antigenic heterogeneity detected by 31D8 antibody binding correlates with functional heterogeneity and may help explain observed functional differences between neonates and adults. Neonatal cord PMNs that traversed polycarbonate micropore filters into the lower compartment of Boyden chambers expressed a greater amount of 31D8 antigen on their surface and had a higher percentage of bright cells compared with PMNs that remained above the filters. Seligmann et al also found that PMNs that traversed micropore filters had a significantly higher percentage of 31D8 bright cells when the cells were labeled with 31D8 before placement in the Boyden chambers.[15] This indicated that up-regulation of 31D8 antigen was not a sufficient explanation for the increased percentage of 31D8 bright cells among the PMNs that traversed the micropore filter. Furthermore, we noted a progressive increase in PMN chemotaxis and 31D8 antibody binding in neonates studied at birth, again at three to 15 days of age and in adults. Although there is insufficient evidence to determine the quantitative contribution of 31D8 subpopulation changes to alterations in chemotactic responses, these results suggest that decreased PMN chemotaxis in neonates may be due in some part to the smaller percentage of highly motile 31D8 bright PMNs found in neonates compared with that of adults.

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Chromosomal Translocations Play a Unique Role in Influencing Prognosis in Childhood Acute Lymphoblastic Leukemia

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Certain types of chromosomal abnormalities have been shown to exert strong independent influence on treatment outcome in acute lymphoblastic leukemia (ALL). To identify the changes most closely associated with prognosis, we analyzed the completely banded blast cell karyotypes of 161 children with this disease. One hundred twenty-five cases had one or more chromosomal abnormalities, with 45 showing translocations. The frequency of translocations was highest (58%) among patients with pseudodiploid karyotypes and lowest (0%) in the hyperdiploid group defined by 51 or more chromosomes. During the maximum 6-year follow-up period, 30 of the 45 patients with a translocation failed therapy, compared with only 27 of the 116 who lacked this feature. Life-table estimates of event-free survival indicate that only 14% of the translocation group will be in complete remission at 3 years. The percentages of failures associated with random and non-random translocations were virtually identical (68% v 65%). When entered in a Cox proportional hazards model with seven other types of chromosomal abnormalities, and then with 11 clinical and laboratory variables of known prognostic value in ALL, translocation emerged as the strongest single predictor of treatment outcome ($P < 0.0001$). The model indicated that translocation increases the risk of treatment failure six times by comparison with the absence of this feature. These findings offer an explanation for the majority of early treatment failures in childhood ALL, including those previously attributed to ploidy classification.

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IDEALLY, prognostic factors should allow unequivocal separation of risk groups at diagnosis so that more effective treatment can be devised for patients likely to fail. Acute lymphoblastic leukemia (ALL) is now curable in more than 50% of children,[1,2] making it possible to concentrate on achieving an optimal risk:benefit ratio for modern therapy. Past reliance on clinical, cytologic, and immunologic factors to classify newly diagnosed patients with ALL has led to relatively diverse systems for assignment of risk categories.[3] Sharpening the focus of patient selection methods would be advantageous as a means of improving the reliability, efficiency, and interpretability of clinical trials.

Chromosomal abnormalities have attracted much recent attention as important prognostic factors in ALL. Secker-Walker et al[4,5] were the first to demonstrate that chromosome number (or ploidy) strongly influences treatment outcome in childhood leukemia patients, a relationship that has subsequently been confirmed by others.[6-8] Among 136 children with ALL, we found that those with modal chromosome numbers of 51 or above had the most favorable treatment responses, whereas those with pseudodiploid karyotypes had the poorest responses.[7] Patients with apparently normal, hypodiploid, or 47–49 chromosomes had intermediate responses. The data generated at the Third International Workshop on Chromosomes in Leukemia,[6] comprising 330 children and adults with ALL, showed that the specific karyotypic patterns provided significant independent prognostic information, both with regard to remission duration and to survival. Bloomfield et al[9] have published data indicating that chromosomal translocations, in general, have an adverse effect on prognosis in ALL.

To assess the impact of leukemia cell karyotype and its component parts on disease-free survival, we analyzed an array of chromosomal and other presenting clinical and laboratory features for 161 children with ALL who had complete G-banding of all chromosomes and adequate follow-up. The results demonstrate that the presence or absence of a translocation conveys the most clinically useful information of all prognostic features examined.

MATERIALS AND METHODS

Patients and Treatment

Of 398 consecutive patients with newly diagnosed ALL admitted to St. Jude Children's Research Hospital from July 1, 1978, to January 1, 1983, 301 had adequate bone marrow samples and successful chromosome studies; 161 of these cases had complete G-banding of all chromosomes. The present analysis was restricted to the latter group to avoid possible underrepresentation of clonal abnormalities. The 161 patients were entered in three institutional clinical trials: 17 in Total Therapy Study IX and 137 in Study X (95 in the standard-risk arm and 42 in the high-risk arm),[7] 7 of the 8 B-cell ALL patients were treated separately (unpublished results, protocol available on request). Although treatment components differed among the three studies and between subdivisions of Study X,[7] times to failure for patients with fully banded chromosomes showed no significant differences by major therapeutic protocol ($P = 0.164$, data not shown); similarly, the clinical features and times to failure for all censored patients ($n = 237$) were comparable to those of the 161 patients in the study group. All patients were enrolled in studies (that included a cell profile analysis) approved by the institution's clinical trials committee. All patients were advised of the procedures and attendant risks, in accordance with institutional guidelines, and informed consent was obtained in each instance.

The percentage of completely banded cases versus the total number of patients admitted to the hospital per year is as follows: 20% (8/41) in 1978, 27% (24/88) in 1979, 34% (30/88) in 1980, 46% (46/100) in 1981, and 58% (53/92) in 1982. These percentages

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do not include cases with partial banding or those in which only ploidy classification was obtained.

The distribution of follow-up times for patients with complete banding was as follows: 22 cases for 0 to 2 years, 51 cases for 2 to 3 years, 40 cases for 3 to 4 years, 27 cases for 4 to 5 years, and 21 cases for 5 to 6 years. Follow-up time was defined as the number of years between the date on-study and the analysis date (ie, August 1, 1984).

Chromosome Analysis

Chromosomes were prepared by a direct bone marrow technique for ALL developed in our laboratory.[10] Marrow cells were collected in RPMI-1640 medium supplemented with 30% fetal calf serum. Only 0.1 mL of sedimented cells was processed per centrifuge tube. Without delay, the cells were exposed to Colcemid (0.06 μg/mL) for 25 minutes, and then to hypotonic KCL (0.075 mol/L) for a total of 32 minutes (including mixing time, standing time, and centrifuging time). They were then fixed in 3:1 methanol-acetic acid (v/v) and slides were prepared by an edging-flaming technique. Appropriate drying of the slides was achieved by natural aging. G-banding was done by treatment with trypsin and staining with Wright's stain.

For each case, 10–35 metaphases (mean, 20) were studied by direct microscopy to determine the modal number and to identify the malignant stem-line. Karyotypes were prepared from photographic prints. Chromosomal abnormalities were classified according to international conventions.[11,12] The definition of an abnormal stem line was that proposed by the Second International Workshop on Chromosomes in Leukemia.[13] A case was considered abnormal if it had an abnormal clone, regardless of the proportion of normal metaphases. In 12 of 13 cases with two leukemic lines, the second line derived from the other through clonal evolution; hence, only the primary line was included in this study. For the single case with two independent stem lines, both lines were included. Because some chromosomal abnormalities appeared in only one to three cases, they were not included in the statistical analysis. These included rings, inversions, and tiny DNA fragments; because all of these occurred in cases with multiple abnormalities, no individual case was excluded.

The abnormalities analyzed were whole chromosome gain or loss, translocation, unidentified DNA addition not included in translocation because a duplication could not be ruled out, DNA loss (deletion) not identified in a translocation, DNA duplication, isochromosome, and unidentified marker. Translocations were designated as "random" or "nonrandom." The random translocations were those appearing only once, and to our knowledge, not previously reported at the time of this study. The nonrandom translocations were those that appeared two or more times and/or have been reported to cluster.

Hematologic and Immunophenotypic Studies

The diagnosis of ALL was based on morphologic criteria of the French-American-British (FAB) group[14] and negative myeloid-associated cytochemical findings.[15] Immunophenotypes, determined by published methods,[16–21] supported the diagnosis in all instances.

Statistical Analysis

Time-to-failure curves were plotted by the method of Kaplan and Meier,[22] and significant differences were determined with the Mantel-Cox statistic.[23] Failure was defined as a lack of response to induction therapy, a relapse during first complete remission, or death due to any cause. Cox regression analysis[24] was used to assess the influence of chromosomal abnormalities and other potentially important prognostic factors on failure rate. This "proportional-hazards" model permits measurement of the contribution of a given covariate to duration of complete remission while simultaneously adjusting for the contributions of other covariates. The prognostic factors studied, in addition to chromosome abnormalities and chromosome number, were log leukocyte count, relapse risk as defined for Total Study X at St. Jude Hospital, mediastinal mass, age, sex, race, cell markers, FAB type, E-rosette formation, and CNS leukemia.

RESULTS

Modal Number of Chromosomes

The distribution of chromosome numbers was typically bimodal,[7] with the first peak occurring at 46 chromosomes (representing mainly patients with pseudodiploid karyotypes) and the second at 55 chromosomes (Fig 1). Each ploidy group appeared to be adequately represented by comparison with findings from an earlier analysis of 136 cases including both partially and fully banded karyotypes.[7] Although the percentage of cases containing a normal karyotype (22%) is not typical of our more recent studies (6%), this group was included in order to obtain the proper perspective in regard to prognosis.

Chromosomal Abnormalities By Ploidy Group

Overall, the most frequent chromosomal abnormalities by case distribution were whole chromosome additions (62 cases), translocations (45 cases), unidentified markers (34 cases), whole chromosome loss (32 cases), and DNA deletions (28 cases). Because of the established relationship between modal chromosome number and prognosis,[4–8] we next determined the distribution of chromosomal changes according to ploidy group. The results (Fig 2) depict structural and numerical differences as well as specific chromosomes involved in translocations. Findings for the hypodiploid group, which comprised only nine cases, did not permit valid conclusions and were omitted from the figure.

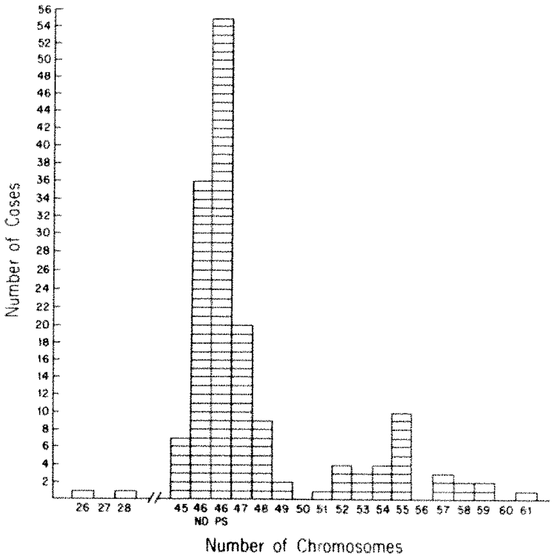

Fig 1. Distribution of modal numbers among 161 cases of childhood ALL. Note the bimodal distribution pattern, with the first peak occurring at 46 chromosomes (pseudodiploid) and the second at 55 chromosomes. ND = normal diploid; PS = pseudodiploid.

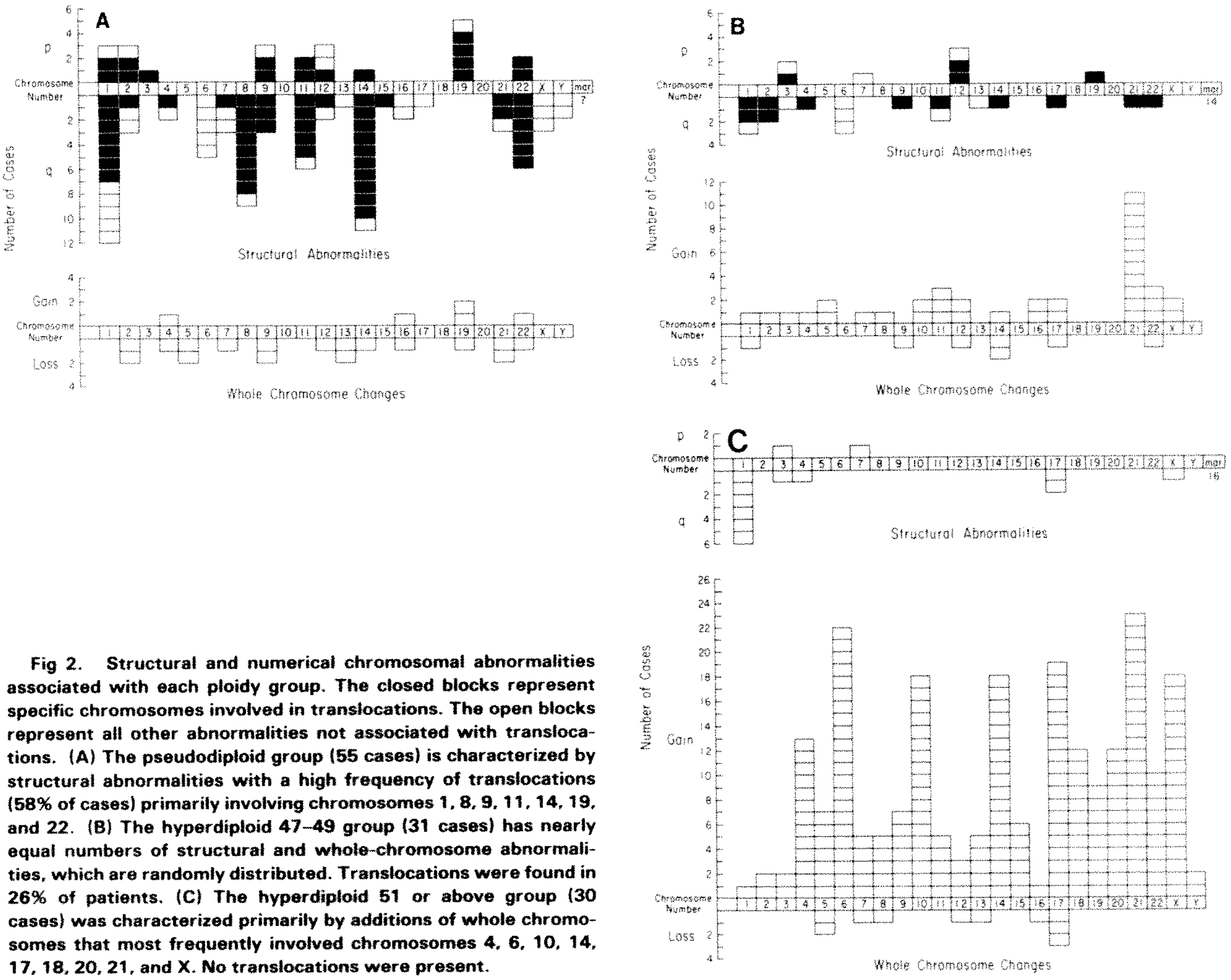

Fig 2. Structural and numerical chromosomal abnormalities associated with each ploidy group. The closed blocks represent specific chromosomes involved in translocations. The open blocks represent all other abnormalities not associated with translocations. (A) The pseudodiploid group (55 cases) is characterized by structural abnormalities with a high frequency of translocations (58% of cases) primarily involving chromosomes 1, 8, 9, 11, 14, 19, and 22. (B) The hyperdiploid 47–49 group (31 cases) has nearly equal numbers of structural and whole-chromosome abnormalities, which are randomly distributed. Translocations were found in 26% of patients. (C) The hyperdiploid 51 or above group (30 cases) was characterized primarily by additions of whole chromosomes that most frequently involved chromosomes 4, 6, 10, 14, 17, 18, 20, 21, and X. No translocations were present.

Briefly, this group contained two near-haploid cases (with 26 and 28 chromosomes) and seven cases with 45 chromosomes containing different karyotypes. Five of the latter cases contained translocations (two nonrandom and three random). Each of the nonrandom translocations was identified as the t(1;19) abnormality specifically associated with pre-B–cell ALL.[25-28]

Pseudodiploid group. All but one of these 55 cases contained structural abnormalities most frequently involving the long arms (q) of chromosomes 1, 8, 11, 14, and 22 (Fig 2A). Translocations were observed in 32 cases (58%). Twenty nonrandom translocations were identified: seven containing the t(8;14) or $14q^+$ abnormality, all with B-cell ALL; six the t(9;22) or $22q^-$; one the t(4;11); four the t(1;19), all with pre-B–cell ALL; and two the t(11;14), both with E^+ T-cell ALL.[25] Twelve different, apparently random translocations were observed. Structural abnormalities not involving translocations were found in 41 other chromosomes, and there were seven banded but unidentified markers. Numerical changes were much less frequent than structural abnormalities and all were random.

Hyperdiploid group, 47–49. In these 31 cases, numerical and structural abnormalities were distributed nearly equally (Fig 2B). The distribution of abnormal chromosomes was random, and when the six cases of Down's syndrome with trisomy 21 were excluded, the frequency of an added chromosome 21 (5 cases) was insignificant. Translocations occurred in eight cases (26%), and included three nonrandom [t(9;22), t(1;19), and t(4;11)] and five random translocations. Comparison of karyotypes containing 47, 48, and 49 chromosomes failed to disclose any related patterns of abnormalities, and there were no similarities between this ploidy group and the group with 51 or more chromosomes (see below).

Hyperdiploid group, 51 or above. This group of 30 cases differed markedly from the other abnormal ploidy groups, with most of the abnormalities involving whole chromosome additions (Fig 2C). Extra chromosomes formed trisomies, with numbers 4, 6, 10, 14, 17, 18, 20, 21, and X added most frequently and number 21 added twice in 12 cases. This apparent nonrandom addition of at least nine chromosomes contributed to the secondary peak of 55 chromosomes apparent in Fig 1. Only one patient in this group had Down's syndrome with trisomy 21, and the leukemic clone contained only that one extra 21 chromosome. Thus, the influence of cases with Down's syndrome on the observations related to chromosome 21 was negligible. Structural abnormalities were relatively infrequent, most often involving 1q in a partial duplication, and included 16 unidentified markers. Translocations were not identified in these 30 cases.

Our most recent studies indicate that translocations do occur in this group, in up to 14% of cases, but this number is still relatively small in comparison to the other ploidy groups. Preliminary results indicate that as in other ploidy groups, the presence of a translocation carries more prognostic import in this setting than does hyperdiploidy. Additional case studies and longer follow-up are needed to confirm this finding.

Treatment Response According to Chromosomal Abnormality

From the preceding analysis, it was clear that a high frequency of structural abnormalities, particularly translocations, distinguishes pseudodiploid cases. This was in contrast to the high frequency of added intact chromosomes and the relative paucity of structural abnormalities, including translocations, in the 51 or above group. Table 1 tests our impression that the presence or absence of a translocation explains prognostic differences among commonly recognized ploidy groups. Within the pseudodiploid and 47–49 groups, the presence of a translocation had a highly significant influence on the frequency of treatment failure. In the pseudodiploid group, only 56% of patients with a translocation achieved a complete remission (CR). The best overall response was found in the group with 51 or more chromosomes, which contained no translocations. When the analysis included only cases without translocations, failure rates among the three ploidy groups showed no significant differences ($P = 0.4246$). Addition of hypodiploid cases without translocations, as well as cases with normal karyotypes, did not alter this finding ($P = 0.8050$, data not shown).

Overall, patients with translocations had strikingly different times to failure compared with those lacking the abnormality ($P < 0.0001$, Fig 3A). Remission induction failure rates also differed: 20% for the translocation group versus 4% for others ($P = 0.002$). All but 1 of the 30 patients with translocations who failed did so within 2½ years, while receiving combination chemotherapy; whereas those without translocations had a more variable clinical course. A comparison of survival times (Fig 3B) emphasized the aggressiveness of disease in cases with translocations. Only two patients

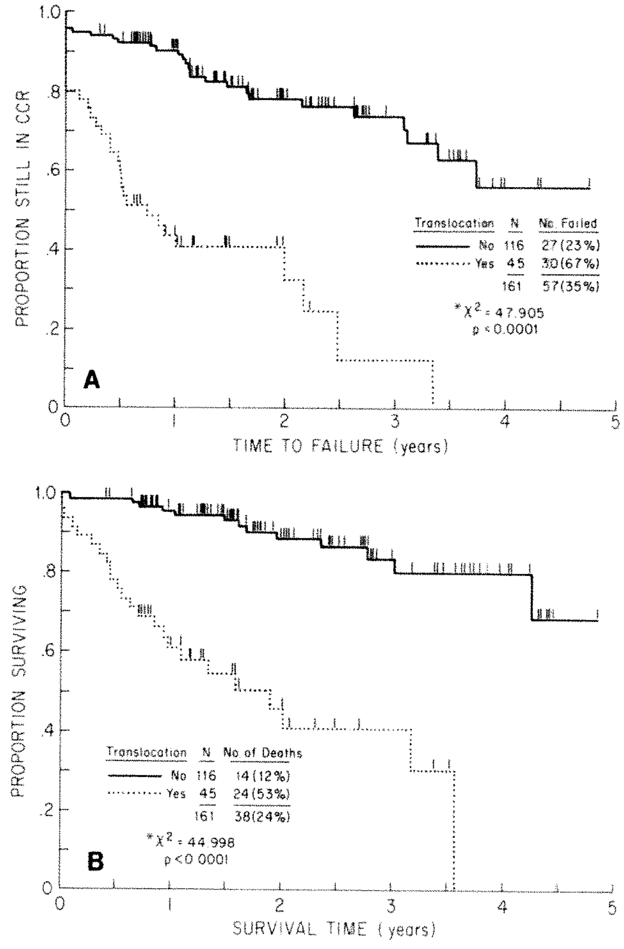

Fig 3. Time to failure (A) and survival time (B) by presence or absence of a translocation. The Kaplan-Meier curves in panel A depict differences in times to failure for the translocation and nontranslocation groups. Remission induction rates differ as indicated by the starting points on the graph at time 0. Nearly all patients with translocations who failed did so by 2½ years or before the end of therapy, whereas in the nontranslocation group the failure pattern is more variable. A similar pattern appears in the analysis of survival times (panel B), indicating the agressiveness of the disease in the translocation group. Of patients with translocations who died, 92% (22 of 24) died before 2½ years.

Table 1. Relationship of Translocation Frequency Within Ploidy Groups to Treatment Outcome

Major Abnormal Ploidy Group	With Translocation			Without Translocation			
	Patients	CR*	Failures†	Patients	CR	Failures	*P* Value‡
Pseudodiploid	32/55 (58%)	18/32 (56%)	24/32 (75%)	23/55 (42%)	20/23 (87%)	6/23 (26%)	<0.001
Hyperdiploid 47–49§	8/31 (26%)	7/8 (88%)	5/8 (63%)	23/31 (74%)	23/23 (100%)	4/23 (17%)	0.009
Hyperdiploid 51 or above	0/30 (0%)	—	—	30/30 (100%)	30/30 (100%)	5/30 (17%)	—

*Complete remission induction.

†No response to induction therapy, relapse during first complete remission, or death due to any cause.

‡From Mantel-Cox comparison of failure rates over time.

§The designation 47–49 is made in this paper because there were no cases with exactly 50 chromosomes.

Table 2. Distribution of Treatment Failures by Category of Translocation

Nonrandom	Failures/No. of Cases	Random*	
t(8;14)(q24;q32) or 14q+	7/8	t(1;11)(p22;q23)†	t(2;16)(p12;p11)
t(9;22)(q34;q11) or 22q−	6/7	t(1;11)(?p33;q25)†	t(7;9)(p?;q?)†
t(4;11)(q21;q23)	1/2	t(1;12)(q25;p12)	t(8;14)(q24;q12)†
t(1;19)(q23;p13.3)	2/7	t(1;14)(p?;q32)†	t(9;11)(p13;q25)
t(11;14)(p13;q13)	1/2	t(1;21)(q12;q22)†	t(9;12)(p22;p12)
		t(2;3)(p14;p14)†	t(11;15)(q13;q25)†
		t(2;3)(q11;p25)	t(12;17)(p12;q12)†
		t(2;9)(?q14;q34)†	t(13;14)(p11;q11)†
		t(2;12)(q14;p12)†	t(21;22)(q21;q12)†
			t(9;10)(q22;q11) t(10;11)(q24;q13) ‡
Total	17/26 (65%)	Total failures/no. of cases:	13/19 (68%)

*Each translocation represents one case, except as indicated (‡).

†Failures.

in this category have lived beyond 3 years from diagnosis despite attempts to secure new remissions.

Table 2 lists all of the translocations identified in the study, categorized as either nonrandom or random. The first group consisted of well-recognized translocations identified in 26 patients; the second comprised 20 different translocations (not previously found in clusters) in 19 patients and involved many different chromosomes. Comparison of failure rates over time, using the Mantel-Cox statistic, disclosed no significant differences between the two groups (P = 0.9078, data not shown).

Multivariate Analysis

Table 3 shows the relative prognostic importance of the eight major categories of chromosome abnormalities, as determined by Cox regression analysis. When the categories were studied singly, translocation exerted the strongest influence on remission duration ($P < 0.0001$), followed in descending order by whole chromosome gain ($P = 0.0165$) and unidentified marker ($P = 0.0578$). After adjustment for translocation, neither variable retained significance, but translocation lost none of its predictive power in the presence of other covariates.

A number of clinical and laboratory features have been found in the past to have prognostic value in childhood ALL.[1] Prominent among these have been leukocyte count, mediastinal mass, age, sex, race, immunophenotype, FAB type, and CNS leukemia. Table 4 lists the distribution of these features among our population of patients separated as: (1) those without a translocation and (2) those with a translocation. In those with a translocation there is a higher frequency of increased leukocyte counts, black race, B-cell phenotype, L3 FAB type, and CNS leukemia. When the eight features were studied in each of the 45 patients with a translocation individually, results indicated that 37 out of 45 (82%) had at least one high-risk feature.

Since the majority of patients with a translocation had one or more "classic" high-risk features, it was important to account for their competing effects on treatment outcome. Thus, the same proportional hazards model was used to determine the influence of translocation on failure rates relative to these well-recognized prognostic factors in ALL (Table 5). In the univariate analysis, translocation, and ploidy emerged as the strongest variables ($P < 0.0001$). Log leukocyte count ($P = 0.0021$) also made a significant contribution, as did cell markers ($P = 0.0378$). When the individual variables were adjusted by translocation, only chromosome number retained significance but at a greatly reduced level. Again, as in Table 3, no covariate added prognostic information to translocation, even when the four next most important factors (log leukocyte count, chromo-

Table 3. Prognostic Strength of Different Chromosomal Abnormalities*

	Univariate Analysis		Covariates Adjusted For Translocation		Translocation Adjusted For Other Covariates	
Abnormality	χ_1^2	*P* Value	$\Delta\chi_1^2$	*P* Value	$\Delta\chi_1^2$	*P* Value
Translocation (n = 45)	33.50	<.0001	—	—	—	—
Whole chromosome addition (n = 62)	5.75	.0165	2.76	.0964	30.52	<.0001
Whole chromosome loss (n = 32)	1.60	.2057	3.40	.0653	35.30	<.0001
DNA addition† (n = 13)	0.15	.7004	1.44	.2296	34.80	<.0001
DNA loss‡ (n = 28)	0.11	.7409	0.41	.5230	33.80	<.0001
Duplication (n = 10)	0.02	.8952	0.31	.5806	33.79	<.0001
Unidentified marker (n = 34)	3.60	.0578	0.50	.4806	30.40	<.0001
Isochromosome (n = 4)	0.56	.4526	0.31	.5772	33.25	<.0001

*By Cox regression analysis.

†Unidentified DNA addition (not included in translocation because a duplication could not be ruled out).

‡Simple deletion (not included in translocation).

Table 4. Distribution of High-Risk Features Between Patients With or Without a Chromosomal Translocation

Feature	Category	Presence of a Translocation	
		No (n = 116)	Yes (n = 45)
Leukocyte count	25 to 100	20	12
(x 10^9/L)	$\geq$100	13	10
Mediastinal mass	Yes	12	4
Age (yr)	<2	6	6
	$\geq$10	28	10
Sex	Boys	76	22
Race	Nonwhite	7	7
Immunophenotype	T-cell	19	7
	B-cell	0	7
FAB type	L2	25	6
	L3	0	7
CNS leukemia	Yes	5	12

some number, relapse risk, and cell markers) were considered as a group (data not shown).

From results of the Cox regression analysis, it was possible to calculate the relative risk of treatment failure associated with each covariate. Patients with a translocation were six times more likely to fail treatment than those without the abnormality. The probability of treatment failure before the end of the first year was much greater for patients with translocations: 0.57 *v* 0.10 for patients lacking the abnormality. Similarly, those with translocations had a much greater probability of dying (0.39 *v* 0.05).

DISCUSSION

In this analysis of fully banded chromosomes from a large series of childhood leukemia patients, we were able to identify translocation as the abnormality most frequently associated with treatment failure. These observations confirm and extend some of the work of the Third International Workshop[6] and of Bloomfield et al.[9] The unusual distribution of translocations, predominantly among the pseudodiploid group, offers an explanation for the prognostic strength assigned to chromosome number in previous studies.[4-8] The relatively small number of translocations in the 51 or above group, first observed by the Third International Workshop on Chromosomes in Leukemia,[6] is consistent with the low failure rate observed in this study (Table 1) and the paucity of other high-risk features previously observed among patients in this category.[7] Moreover, these hyperdiploid ALL cases had an apparent nonrandom pattern of whole chromosome additions, forming trisomies, that was not seen in the 47–49 or other ploidy groups; cases with exactly 50 chromosomes have not been observed by us at diagnosis. These facts provide compelling evidence that the 51 or above group is

Table 5. Prognostic Strength of Translocation Relative to Other Recognized Prognostic Factors in ALL*

Feature	Total Cases	Distribution of Subgroups	Univariate Analysis		Covariates Adjusted for Translocation		Translocation Adjusted for Other Covariates	
			χ^2_1	*P* Value	$\Delta\chi^2_1$	*P* Value	$\Delta\chi^2_1$	*P* Value
Translocation	161	45 (yes) 116 (no)	36.11	<.0001	—	—	—	—
Log leukocyte count†	161		9.50	.0021	3.35	.0672	29.95	<.0001
Chromosome no.‡	161	55 (pseudo) 30 (hyper $\geq$51) 76 (others)	22.24	<.0001	6.07	.0481	19.94	<.0001
Relapse risk§	161	116 (standard) 45 (high)	3.46	.0630	0.62	.4302	33.28	<.0001
Mediastinal mass	161	16 (yes) 145 (no)	1.12	.2893	1.87	.1717	36.85	<.0001
Age (yr)	161	112 (2-9) 49 (others)	2.55	.1103	1.59	.2072	35.15	<.0001
Sex	161	98 (male) 63 (female)	0.86	.3550	0.26	.6069	35.52	<.0001
Race	161	147 (white) 14 (black)	2.17	.1407	1.08	.2987	35.02	<.0001
Cell markers	157	26 (T) 7 (B) 124 (others)	4.32	.0378	2.68	.1014	31.88	<.0001
FAB type	160	122 (L1) 31 (L2) 7 (L3)	1.09	.2967	1.00	.3164	34.37	<.0001
E-rosette formation	148	18 (yes) 130 (no)	0.06	.8137	0.02	.8778	31.67	<.0001
CNS leukemia	160	17 (yes) 143 (no)	1.14	.2861	0.62	.4308	36.30	<.0001

*By Cox regression analysis.

†Used as a continuous variable in the Cox regression analysis.

‡Pseudodiploid *v* hyperdiploid $\geq$ 51 *v* all others.

§High risk = leukocyte count > 100 × 10^9/L, mediastinal mass, CNS involvement at diagnosis or E^+ blasts; standard risk = absence of high-risk features.

chromosomally distinct, as first suggested by Kaneko et al,[8] and should be regarded as a unique subset of patients with a better prognosis, except for patients whose karyotypes contain a translocation.

The time-to-failure comparisons in this study indicate that in childhood ALL, translocations as a group convey a poor prognosis. This includes many of the so-called random translocations, which may prove to have a nonrandom (or specific) distribution when larger numbers of cases are studied, as suggested by the finding that the t(11;14) and the t(1;19) correlate with T-cell and pre-B–cell ALL, respectively.[25] Although at least two translocations in other malignancies have been shown to convey a relatively good prognosis [the t(14;18) in follicular small cleaved cell lymphoma and the t(8;21) in ANLL], there has been no similar subgroup identified for ALL in the studies of the Third International Workshop,[6] in those of Bloomfield,[9] or in our studies. Future studies however, may reveal some heterogeneity of response among translocations as additional new subgroups are identified. Likewise new therapies may partially or completely alter the correlation between translocation and prognosis.

It now seems likely that the recognized consistent translocations affect chromosome segments involved in malignant transformation.[29–31] In the present study, the high rate of early failure and poor overall survival of patients with translocations suggest an additional effect, ie, one in which translocations are associated with clinical drug resistance. How this might occur is unclear. One attractive possibility is that translocations increase the proliferative rate of malignant cells, leading to more aggressive disease. Patients with B-cell ALL, characterized by the t(8;14) abnormality or one of the variant forms, have a very poor prognosis and a high leukemic cell proliferative rate.[25] Yet, data reported by us for other immunophenotypes of ALL do not support a proliferative advantage for all leukemic cells with a translocation.[25] To the contrary, in that study, T-cell ALL cases with the 11;14 translocation had a significantly lower percentage of cells in S phase than did those without the translocation. Alternatively, drastic genetic reassortment, typified by translocations, could alter gene expression in ways that might decrease the penetration of drugs into cells or perturb their metabolic fate once they are inside cells.

This study indicates that a translocation is the element within the total karyotype that would be most useful to include in the pretreatment risk assignment for the design of clinical trials in ALL. Chromosome number, or ploidy, appears to be the second strongest feature. It is now possible, in ALL, to obtain analyzable chromosomes and identify abnormal leukemic clones in nearly all cases. Several techniques for the study of bone marrow cells[32–36] are providing good results. In addition, an improved direct technique designed especially for chromosome preparation in ALL is now available, and this method provides a high yield of successfully banded cases, if carefully performed.[10] In our recent experience, it has also allowed identification of chromosomally abnormal lines in a very high percentage of cases analyzed (94%). Recognition of the 51 or above group, especially, may also be achieved by flow cytometric methods and these studies may be used together to confirm and complement each other.[37,38] However, the identification of a translocation depends upon chromosome analysis. Since it may not be possible *in every case* to obtain complete banding and thus recognize or rule out a translocation, the use of either translocation or ploidy may in some situations provide a practical assessment of risk of treatment failure in ALL. We suggest that the presence of any translocation can be used routinely to define high-risk cases. At our institution this karyotypic feature is included with the flow cytometrically determined DNA index, white blood cell count, age, and race for allocation of patients to discrete treatment groups.

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Monoclonal Antibodies Against SU-DHL-1 Cells Stain the Neoplastic Cells in True Histiocytic Lymphoma, Malignant Histiocytosis, and Hodgkin's Disease

By Su-Ming Hsu, Mark D. Pescovitz, and Pei-Ling Hsu

Three murine monoclonal antibodies, named 2H9, 1E9 and 1A2, were produced after immunization of BALB/c mice with cells of the SU-DHL-1 cell line from a true histiocytic lymphoma. In frozen sections from various lymphomas, 2H9 and 1A2 selectively stained the cell membranes of neoplastic cells in true histiocytic lymphoma and Hodgkin's disease. Antibody 1E9 stained the nuclear membranes of the tumor cells in true histiocytic lymphoma and malignant histiocytosis. No staining was seen in 56 cases of B and T cell lymphoma. Several tissue culture cell lines, including T cell acute lymphoblastic leukemia and pre-B cell lines, were not stained. With 2H9, however, a positive reaction was noted for two Epstein-Barr virus (EBV)-positive African Burkitt's lymphoma cell lines (Daudi and P3HRI), one human T cell lymphoma/leukemia-virus-positive cell line (HUT 102), and one EBV-transformed normal B lymphoblastoid cell line (RPMI 8057). In normal lymphoid tissues, 2H9 and 1E9 reacted with the nuclear membranes of histiocytes and interdigitating reticulum cells, whereas 1A2 stained only rare cells of an unknown type. All three antibodies failed to react with B or T cells in frozen tissue sections of normal lymphoid tissues. The use of these three antibodies should facilitate the diagnosis of histiocyte and interdigitating reticulum (IR) cell-related neoplasms, namely, true histiocytic lymphoma, malignant histiocytosis, and Hodgkin's disease. True histiocytic lymphoma and Hodgkin's disease exhibit similar reactivities with these three and with two other monoclonal antibodies (HeFi-1 and Tac), suggesting that these two types of lymphoma are related. In contrast, malignant histiocytosis was negative for 2H9, 1A2, Tac, and HeFi-1. The difference in the phenotypic expression of true histiocytic lymphoma and malignant histiocytosis indicates that they are two different disease entities.

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IT IS NOW recognized that the so-called diffuse "histiocytic" lymphomas (DHL) of the Rappaport classification represent a heterogeneous group of neoplasms originating from B cells, T cells, and true histiocytes.[1] In 50% to 60% of DHL, the cells have characteristics of B lymphocytes; in 5% to 15%, those of T lymphocytes; and in only 5%, those consistent with monocytes or true histiocytes.[1,2] In approximately one-third of the cases, the cells do not have B cell, T cell, or histiocyte characteristics; they have been called "null" cells. With the development of new monoclonal antibodies (MoAbs) and sophisticated immunotyping techniques, however, some of these cases are now being reclassified in either the B or the T cell lineage.

The diagnosis of a B or T cell lymphoma is not difficult, because a large panel of MoAbs specific for either B or T cells is available. However, owing to the lack of antibodies specific for human histiocytes or histiocytic lymphoma cells, the confirmation of a true histiocytic lymphoma is usually not as clear-cut. Although a large number of antibodies against human monocytes are currently available, most of these antibodies do not react with histiocytes or histiocytic lymphoma cells,[3] despite the view that histiocytes and monocytes are closely related.

In this study, we sought to develop histiocyte-specific MoAbs by using cells of the SU-DHL-1 line as the immunogen. The SU-DHL-1 cell line was derived from a patient with histiocytic lymphoma.[4] These cells consistently failed to express T or B cell markers, even after they were induced by phorbol ester (TPA), retinoic acid, dimethylsulfoxide (DMSO), or heme.[3] The SU-DHL-1 cells lack immunoglobulin gene rearrangements.[5,6] They demonstrate cytoplasmic acid phosphatase and nonspecific esterase (ANAE) activities, and they can be stained with a MoAb, anti-Leu M5, that normally reacts with monocytes and histiocytes.[3,7,8] Thus, there is little doubt that SU-DHL-1 is a true histiocytic lymphoma cell line. We report here the production of three MoAbs against the SU-DHL-1 cells. These MoAbs may facilitate the diagnosis of true histiocytic lymphoma (THL) and other histiocyte and interdigitating reticulum (IR) cell-related neoplasms.

MATERIALS AND METHODS

Immunization, cell fusion, and establishment of hybridoma lines. Male BALB/c mice, 8 to 10 weeks old, were immunized intraperitoneally (IP) with 1 × 10^7 SU-DHL-1 cells on days 1, 8, and 15. The fusion protocol was that described by Ozato and colleagues.[9] In brief, on day 18, mouse spleen cells were harvested under sterile conditions and fused with the non-Ig-producing variant myeloma line SP2/0.Ag14 of BALB/c origin. Hybrids were selected in hypoxanthine-aminopterin-thymidine medium. Ten to 14 days after fusion, supernatants from growth-positive wells were screened for binding to SU-DHL-1 cells by the immunocytochemical technique described below. Hybrids of interest were cloned at 1 or 0.5 cell per well on feeder layers of irradiated rat fibroblasts. Ascites was produced by injection of 1 to 2 × 10^6 hybridoma cells IP into BALB/c mice primed with pristane (0.5 mL IP) at least 5 days before the inoculation.

Immunoglobulin (Ig) class typing. The Ig class of the MoAb was determined by the Ouchterlony double-diffusion technique, with class-specific anti-mouse Ig purchased from Litton Bionetics (Kensington, Md).

Preparation of human monocyte-enriched populations. Peripheral blood lymphocytes (PBL) were obtained by Ficoll/Hypaque separation (LSM: Litton) of heparinized whole blood. A monocyte-enriched population was obtained by the method of Falkoff and co-workers.[10] In brief, peripheral blood mononuclear cells were first E rosette-depleted and then incubated with anti-Leu1 MoAb. Rab-

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bit serum was added as a source of complement to lyse cells to which anti-Leu 1 was bound. The enriched population contained 30% to 50% monocytes, which can easily be distinguished from B lymphocytes by the structure of their nuclei. For immunocytochemical staining, the cells were prepared as cytospin smears.

Tissues and cells. Frozen sections of normal lymphoid tissues (tonsils and lymph nodes) and nonlymphoid tissues were prepared as described previously.[11,12] In addition, samples from 29 B cell lymphomas, 27 T cell lymphomas, 15 cases of Hodgkin's disease, 6 THLs, and 6 cases of malignant histiocytosis were obtained for evaluation of the specificity of the antibodies. The diagnosis of B or T cell lymphoma was confirmed by reaction with B or T cell-specific antibodies (Leu 14, B1, lyt3, Leu 1, Leu 2a, Leu 3a, Leu 4, etc.). The diagnosis of THL was based on the following: (a) cytoplasmic acid phosphatase and ANAE activity was abundant; (b) there was no reactivity with any of the B or T cell-specific antibodies; (c) in short-term cultures, the neoplastic cells remained negative for B or T cell markers after induction with TPA, retinoic acid, or heme; (d) the neoplastic cells were capable of phagocytosis of latex particles; and (e) the neoplastic cells formed EA or EAC rosettes. In addition, the diagnosis of malignant histiocytosis was based on its clinicopathologic characteristics, as described by Byrne and colleagues[13] and Scott and colleagues.[14]

The cells were maintained in suspension culture in 10% fetal calf serum (FCS) in RPMI 1640 medium (GIBCO, Grand Island, NY). The SU-DHL-1 cell line was provided by Dr A. Epstein of Northwestern University, Chicago. The Peer, JM, CCRF-H-SB2, and RPMI 8057 cell lines were provided by Dr B. Seon of the University of New York, Buffalo. Other cell lines (listed in Table 1) were obtained from Biotech Research Laboratory, Rockville, Md.

Immunocytochemical technique. The ABC immunoperoxidase technique was applied as described earlier.[15,16] In brief, sections or smears from the various tissues or cells were fixed in acetone for 5 minutes at room temperature. After being washed in Tris-buffered saline (0.01 mol/L of Tris, pH 7.6, 0.15 N NaCl), slides were incubated with MoAbs, followed by biotin-labeled horse antimouse Ig and avidin-biotin-peroxidase complex. Finally, the sections or smears were developed in a peroxidase substrate containing diaminobenzidine, nickel chloride, and H_2O_2. No attempt was made to block endogenous peroxidase activity in eosinophils. The staining of eosinophils, however, did not interfere with the interpretation of results. An immunofluorescence staining method with fluorescein-labeled rabbit anti-mouse Ig was used for detection of the distribution of antigens in HL-60 cells because the localization of endogenous peroxidase activity at the paranuclear region might interfere with the determination of antigens (2H9 and 1E9) in the same area. In some tissues, such as liver, kidney, and pancreas, intrinsic biotin was blocked before immunostaining.[17]

Characterization of specific antigens. For the detection of 2H9, ~5 × 10^7 cultured SU-DHL-1 cells were washed twice with RPMI medium and then lysed on ice for 5 minutes in a buffer containing 20 mmol/L of Tris-HCl, pH 7.2, 0.15 N NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 10 mmol/L of EDTA, 1 mmol/L of phenylmethyl sulfonyl fluoride (PMSF), and 1% aprotinin (Boehringer Mannheim, Indianapolis). The cell extract was clarified by spinning it for 5 minutes in a microcentrifuge. For the detection of 1E9, stepwise extractions of proteins were carried out with: (a) 1% NP40 in Tris-saline, 1 mmol/L of PMSF; (b) 0.6 mol/L of potassium acetate, pH 5.5, 1 mmol/L of PMSF; and (c) 2 mol/L of potassium acetate, 5 mol/L of urea, 10 mmol/L of Tris, pH 7.5, and 1 mmol/L of PMSF. The concentrations of proteins in the extracts were determined by the method of Bradford.[18] For immunoblots, proteins in Laemmli sample buffer (15 μg per lane) were loaded on a 10% SDS polyacrylamide gel and electrophoresed.[19] Before transfer, the gel was soaked in 4 mol/L of urea and 0.1 mol/L of DTT for 20 minutes and then in transfer buffer (20% methanol, 14.4 g/mL of glycine, 3 g/mL of Tris) for 10 minutes. BA 85 nitrocellulose (0.2 μm, Scheicher and Schull) was used as the blotting membrane. For the detection of total transferred proteins, one strip of nitrocellulose was stained with 0.5% fast green in 20% methanol and 5% acetic acid, and then destained. For detection with MoAbs, the filter was first blocked with normal horse serum (1:200 dilution, from Vector stain ABC kit) in a buffer containing 3% nonfat dry milk in TS (20 mmol/L of Tris, pH 7.6, 0.15 N NaCl) for 30 minutes. After being washed with TS plus 0.1% Triton X-100 (TST, Research Products International, Elk Grove, Ill), the filter strips were incubated with MoAbs (one per strip) for 3 hours and then treated as follows: (a) three washes with TST, (b) 30-minute incubation with biotin-labeled horse anti-mouse (1:200) reagent, (c) three washes with TST, or (d) 30-minute incubation with avidin-biotin-peroxidase complexes (Vector ABC). After a last washing with TST, the filter strips were developed with 4-chloro-1-naphthol (stock 3 mg/mL in methanol, diluted 1:5 in PBS prior to use) and H_2O_2.

Table 1. Reactivities of the Three Monoclonal Antibodies Studied With Lymphoma or Normal B Cell Lines

Cell Line	2H9	1A2	1E9
T cell lymphoma			
HUT-78	–	–	–
HUT-102	+ (m)	–	–
Burkitt's lymphoma			
Raji	–	–	–
Daudi	+ (gr)	–	–
P3HR-1	+ (gr)	–	–
B lymphoblastoid cell			
RPMI 8057	+ (m)	–	–
Monocyte/histiocyte lymphoma			
U-937	5% to 10% + (nm)	–	5% to 10% + (nm)
SU-DHL-1	+ (m)	+ (m)	+ (c)
HL-60*	+ (nm)	–	+ (nm)

m, cell membrane; gr, granular; nm, nuclear membrane; c, cytoplasm.

Other virus-negative B or T lymphoma cell lines, including KM-3/REH, NALM-16, NALM-1JR, HPB-NULL, NALL-1, Peer, JM, Molt-3, Molt-4, CEM, Jarka, and CCRF-HS-B-2 exhibit no reactivity with these three antibodies.

*The staining pattern of HL-60 cells remained the same when the cells were induced by exposure to TPA (2 ng/mL) for 48 hours.

RESULTS

Identification of hybridoma supernatants binding to SU-DHL-1 cells. Immunocytochemical assay of supernatants from the growth-positive wells yielded 54 cultures (11%) which produced antibodies capable of staining SU-DHL-1 cells on cytospin slides. The positive, undiluted supernatants were rescreened with frozen sections of normal tonsils so that their reactivities with normal lymphoreticular cells could be determined. Antibodies that reacted with all lymphoid cells or that produced high background or nonspecific (nondiscriminative) staining were eliminated. Three of the supernatants were selected based on their strong reactivity with SU-DHL-1 cells, but minimal reactivity with normal lymphoid tissues. The corresponding cells were expanded and subcloned at 0.5 cell per well, and the MoAbs were named 2H9, 1E9, and 1A2. Both 2H9 and 1A2 stained the cell membranes of SU-DHL-1 cells, whereas 1E9 elicited an intracytoplasmic granular reaction in the juxtanuclear region.

The isotype of all three MoAbs was determined to be IgM κ by the Ouchterlony double-diffusion technique.

Binding of 2H9, 1E9, and 1A2 to peripheral blood mononuclear cells and normal lymphoid tissues. None of the three MoAbs reacted with B or T lymphocytes in the peripheral blood or in lymphoid tissues such as lymph nodes, tonsils, and spleens.

Antibodies 1E9 and 2H9 stained monocytes in peripheral blood. The former elicited an intracytoplasmic punctate reaction (Fig 1A), whereas the latter produced nuclear membrane staining of variable intensity (Fig 1D); 1E9 also reacted with the cytoplasm of granulocytes (Fig 1A, arrow), whereas 1A2 did not react with either monocytes or granulocytes.

In normal lymphoid tissues, including tonsils and lymph nodes, 1E9 and 2H9 were very similar in their staining pattern; both reacted with the nuclear membranes of histiocytes and of IR cells (Fig 1B and E). We confirmed this reactivity with IR cells by examining lymph nodes from two patients with dermatopathic lymphadenitis, which contained abundant IR cells (Fig 1C). Antibody 1A2 reacted only with rare cells scattered in the lymphoid tissue (Fig 1F). The nature of the 1A2-positive cells is not known; however, some appeared to be tingible-body macrophages in the germinal centers.

Binding of 2H9, 1E9, and 1A2 to tissue culture cell lines. Table 1 shows the reactivities of 2H9, 1E9, and 1A2 with human leukemia cells, lymphoma cells, and normal cell lines. 1A2 did not bind to any of the cells except SU-DHL-1, whereas 1E9 and 2H9 reacted with monocyte/granulocyte/histiocyte cells of lines such as U-937, SU-DHL-1, and HL-60. These reactivities were not unexpected, because 2H9 and 1E9 reacted with monocytes/histiocytes in normal lymphoid tissues (Fig 1A and D); 2H9 also reacted with several virus-positive cell lines. These include: (a) the human T-cell leukemia/lymphoma virus (HTLV)-positive line, HUT 102; (b) two EBV-positive African Burkitt's lymphoma cell lines, Daudi and P3HR1; and (c) an EBV-transformed B lymphoblastoid cell line, RPMI 8057. The staining of 2H9 and 1A2 with the cell membrane of SU-DHL-1 cells was confirmed by flow cytometry and immunoelectronmicroscopy.

Binding of 2H9, 1E9, and 1A2 to lymphomas. Table 2 shows the reactivities of the MoAbs with lymphoma cells in frozen sections when the ABC immunohistochemical technique was used. None of the antibodies stained B or T cell lymphomas (56 cases were studied). Both 2H9 and 1A2 reacted with the cell membrane of Hodgkin's mononuclear cells and Reed-Sternberg cells (H-RS cells) in all 16 cases of

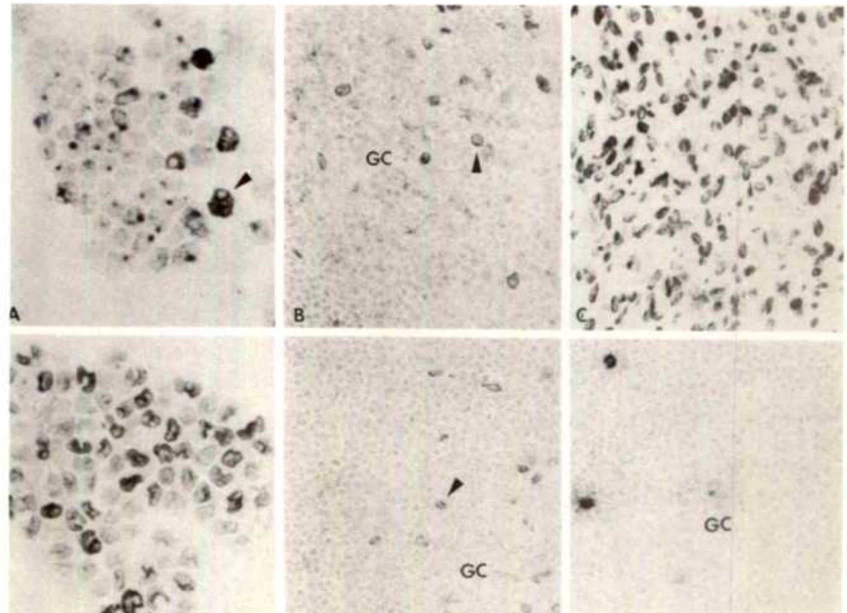

Fig 1. **(A) 1E9 staining of a smear of a monocyte-enriched fraction of PBL, (B) a frozen section of a tonsil, and (C) a frozen section of a lymph node from a patient with dermatopathic lymphadenitis. 1E9 shows an intracytoplasmic granular staining pattern in monocytes. It also reacts with granulocytes (arrow, A). In frozen sections, 1E9 stains the nuclear membrane of histiocytes (arrow, B) and (C) of interdigitating reticulum cells. (D) 2H9 staining of a smear of a monocyte-enriched fraction of PBL and (E) a frozen section of a tonsil. Note difference in staining patterns of monocytes with 2H9 (D) and 1E9 (A). The histiocytes in germinal centers (GC) (arrow, E) are stained by 2H9; (F) 1A2 reacts with rare cells in a section of tonsil (A,D ×400; B,C,E,F ×250.).**

Table 2. Reactivities of Monoclonal Antibodies With Lymphomas

Lymphoma	No. of Cases	2H9	1A2	1E9
B cell lymphoma*	29	–	–	–
T cell lymphoma†	27	–	–	–
True histiocytic lymphoma	6	+	+	+*
Malignant histiocytosis	6	–	–	+*
Hodgkin's disease	16			
Nodular sclerosis	11	+	+	–
Mixed cellularity	4	+	+	–
Lymphocyte predominant	1	+	+	–

*The B cell lymphomas examined include well-differentiated lymphocytic lymphoma (5); intermediately differentiated lymphoma (3); follicular lymphoma, poorly differentiated (5); follicular lymphoma, mixed cellularity (3); small-cell undifferentiated, non-Burkitt's (3); and diffuse large cell lymphoma (10).

†The T cell lymphomas examined included mycosis fungoides (10), peripheral T cell lymphoma (15), and lymphoblastic lymphoma (2).

Hodgkin's disease (Fig 2B). The staining intensity was greater with 2H9 than with 1A2. Although 1E9 failed to stain H-RS cells in tissue sections, it reacted with H-RS cells in short-term cultures, showing an intracytoplasmic granular staining pattern (data not shown).

In all six cases of THL studied, 2H9 and 1A2 stained the cell membrane (Fig 2) and 1E9 stained the nuclear membrane (Fig 3) of the neoplastic cells. In six cases of malignant histiocytosis, 1E9 also stained the nuclear membrane of the neoplastic cells (Fig 3B), whereas 1A2 and 2H9 were negative.

Staining of normal nonlymphoid tissues. Table 3 summarizes the results of staining in various normal, nonlymphoid tissues: 1A2 did not react with any of the tissues examined, and no reactivity with 2H9 was detected except in the cytoplasm of the squamous epithelium in tonsils and in the nuclear membrane of endothelial cells. In contrast, 1E9 stained the nuclear membrane of many nonlymphoid cells, such as the epithelial cells in the thyroid and prostate, as well as endothelial cells.

Identification of the antigens recognized by 2H9, 1E9, and 1A2. Cells from SU-DHL-1 cultures were lysed with detergent or extracted with high salt and urea, and the proteins recognized by the MoAbs were analyzed by the immunoblot technique, as described in the Materials and Methods section. Figure 4 shows that 2H9 recognized a protein of 110 kd in the detergent lysate of SU-DHL-1 cells (Fig 4). A control MoAb, M318, reproducibly recognized a protein of 37 kd, but 1A2 and 1E9 failed to identify any specific protein from the same cell lysate. An alternative extraction procedure with 2 mol/L of potassium acetate and 5 mol/L of urea revealed a 61-kd protein that was recognized by 1E9 (Fig 5). This protein was not present in significant amounts in the 1% NP40 lysate or in the 0.6 mol/L of potassium acetate extract of the SU-DHL-1 cells.

DISCUSSION

Three murine MoAbs, 2H9, 1A2, and 1E9, were produced after immunization of BALB/c mice with the SU-DHL-1 THL cell line. None of the three MoAbs reacted with B cell or T cell lymphomas in frozen tissue sections. They appear to be useful for the diagnosis of histiocyte or IR cell-related lymphomas. However, the diagnostic usefulness of these new MoAbs resides in the differential staining patterns which they exhibited. MoAbs 2H9 and 1A2 stained the cell membranes of neoplastic cells from patients with THL and Hodgkin's disease, whereas IE9 stained the nuclear membranes of the tumor cells in malignant histiocytosis and THL.

The discovery (with 2H9 and 1A2) of a common phenotype between THL cells and H-RS cells is not totally unexpected, however, inasmuch as H-RS cells have been shown to display features of histiocytes/macrophages.[20,21] In this study, despite the difference in the subcellular distribution of antigens (ie, cell membrane in H-RS cells *v* nuclear membrane in histiocytes/IR cells), the reactivity of 2H9 provides additional evidence for a histiocyte/IR cell lineage of H-RS cells.[22]

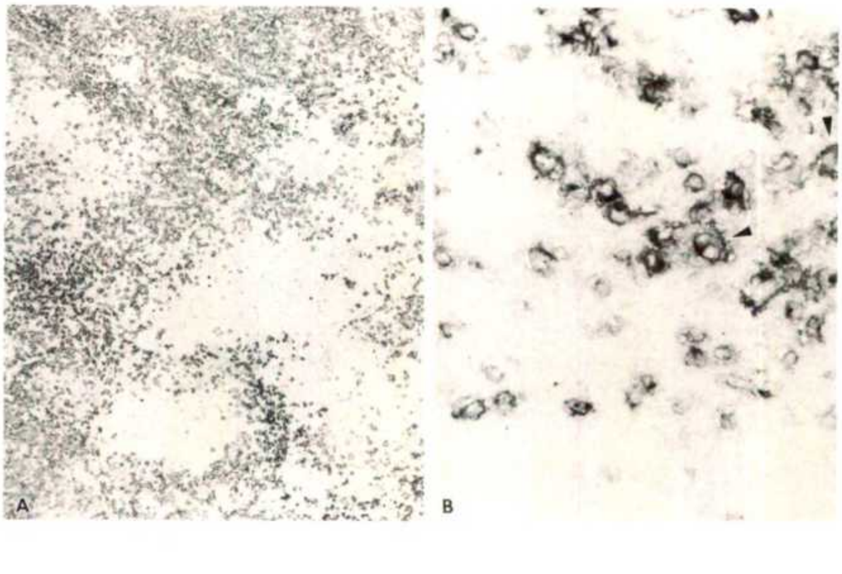

Fig 2. Frozen sections from (A) true histiocytic lymphoma and (B) Hodgkin's disease, stained with 2H9, showing an intense membrane reaction in tumor cells. Arrow in B indicates Reed-Sternberg cells (A and B ×160).

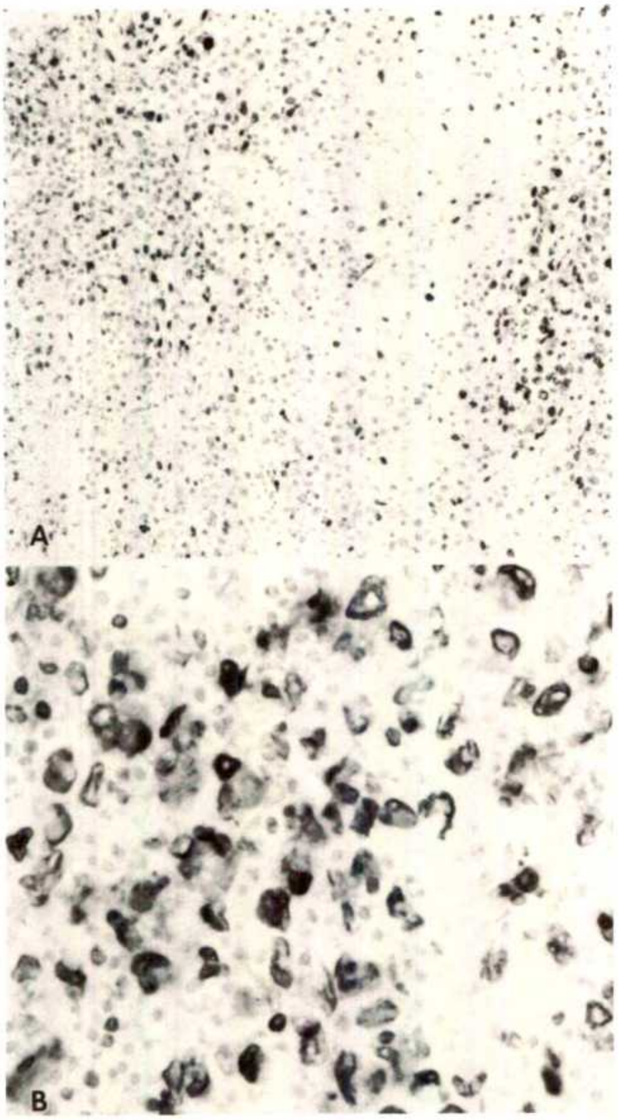

Fig 3. **Frozen sections from (A) true histiocytic lymphoma and (B) malignant histiocytosis. Both stained with 1E9, with staining localized to the nuclear membranes of tumor cells. (A ×160, B ×400).**

Table 3. Reactivities of Monoclonal Antibodies With Normal, Nonlymphoid Tissues

	2H9•	1E9•	1A2
Heart			
cardiac muscle	–	–	–
Lung			
alveolar macrophages	+	+	–
Tonsillar squamous epithelium†	+	+	–
Thyroid	–	+	–
Liver (hepatocytes)	–	+	–
Pancreas			
Acini	–	+	(+) Extracellular staining‡
Islets	–	–	–
Gastrointestinal epithelium	–	+	–
Kidney, tubular epithelium	–	+	–
Prostate†	–	+	–
Fallopian tube	–	+	–
Endothelial cells	–	+	–
Testis, seminiferous tubules	+	–	–
Skin, Langerhans cells	–	–	–

•Nuclear membrane staining.

†Weak cytoplasmic staining is also present.

‡Extracellular staining.

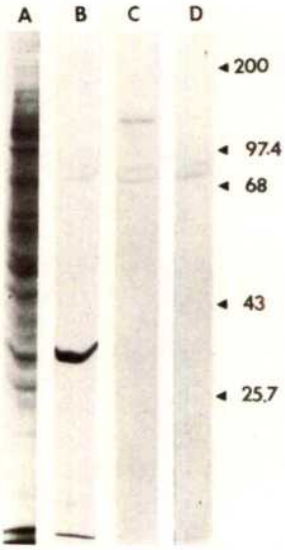

Fig 4. **Immunoblot detection of antigens. Detergent lysate of SU-DHL-1 cells was electrophoresed on a 10% sodium dodecyl sulfate gel, electroblotted onto a nitrocellulose filter membrane, and reacted with specific monoclonal antibodies. Lane A, total protein on filter stained with fast green; lane B, immunoblot with control M318; lane C, immunoblot with 2H9; and lane D, immunoblot with 1A2. The positions of mol wt markers are shown on the right.**

In contrast to the cells in THL, the neoplastic cells of malignant histiocytosis do not express 2H9 and 1A2, but do express 1E9. In recent years, there has been much dispute as to whether THL and malignant histiocytosis are two distinct disease entities. These two lymphomas present a similar histological picture, but differ in their clinical course. The present study indicates that THL cells and malignant histiocytosis cells can be distinguished on the basis of the differential expression of 2H9, 1A2, and 1E9. In other studies, we have also shown that the neoplastic cells of malignant histiocytosis may express OK M1, Mo2, or Leu M5, especially when the malignant histiocytosis cells are induced by a phorbol ester. THL cells, in contrast, do not express these monocyte markers, even with TPA induction.[3]

The difference in the subcellular distribution of the three

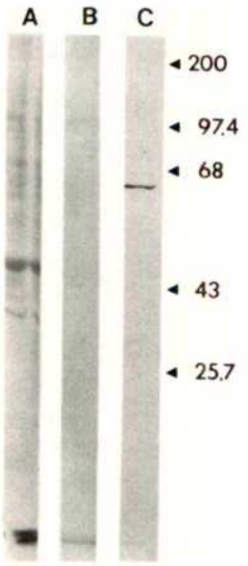

Fig 5. Immunoblot detection of antigen 1E9. An extract (2 mol/L of potassium acetate and 5 mol/L of urea) of SU-DHL-1 cells was electrophoresed on a 10% sodium dodecyl sulfate gel, electroblotted onto a nitrocellulose filter membrane, and reacted with specific monoclonal antibodies. Lane A, total protein on filter stained with fast green; lane B, immunoblot with 1A2; and lane C, immunoblot with 1E9. The positions of mol wt markers are shown on the right.

antigens is of interest. For example, 2H9 appears to react with the cell membranes of H-RS cells and SU-DHL-1 cells, whereas in U-937 (a malignant histiocytosis cell line) and HL-60 (a promyelocytic cell line), a nuclear-membrane staining pattern was observed. We have also found that 1E9 reacts with the nuclear membrane of histiocytes, IR cells, THL cells, and malignant histiocytosis cells in tissue sections, whereas it reacts mainly with a localized area of the cytoplasm of "cultured" SU-DHL-1 or H-RS cells (1E9 did not stain H-RS cells in sections). Staining of U-937 cells with 1E9 normally is observed on the nuclear membrane of ~5% to 10% of these cells, but the 1E9 staining becomes cytoplasmic when the cells are induced with TPA.[3]

Either synthesis of new proteins or redistribution of existing proteins may be responsible for the altered staining pattern. These events may be related to the difference in the status of differentiation or in the cellular environment between cells in cultures or in tissues or between tumor cells and normal cells. We do not know whether 2H9 antigens located in the nuclear membrane and cell membrane are identical. Although some virus-infected cells express viral proteins on nuclear and cell membranes simultaneously, the 2H9, 1A2, and/or 1E9 determinants are not likely to be viral, because they exist in normal histiocytes and IR cells. In animal cells, the outer nuclear membrane is continuous with the endoplasmic reticulum (ER). Like rough ER, the outer nuclear membrane is often studded with ribosomes engaged in protein synthesis. Furthermore, the perinuclear space is continuous with the lumen of the ER. Both the perinuclear space and the outer nuclear membrane can be regarded as a small, specialized region of ER. Therefore, the presence of antigens on the nuclear membrane is not totally unexpected. Future studies of anomalous antigen distributions of these lymphoma cells may provide an understanding of the neoplastic process.

Although both 2H9 and 1E9 react with the nuclear membranes of histiocytes and IR cells, these two antibodies recognize different antigens: 2H9 recognized a 110-kd antigen isolated from SU-DHL-1 cells, whereas 1E9 recognized a 61-kd antigen that was identified in the high salt and urea extract of cells. It should be noted that although 1E9 stains the cytoplasm of SU-DHL-1 cells, the staining is localized. Because this 61-kd protein cannot be extracted with NP-40 or 0.6 mol/L of potassium acetate, it appears that the 1E9 molecules are not soluble and do not diffuse freely in the cytoplasm. It is likely that 1E9 is tightly associated with the cytoskeleton or with a membrane component in the cytoplasm of SU-DHL-1 cells. Whether this 61-kd protein originates in the nuclear membranes of the precursor cells (such as histiocytes or IR cells which show nuclear membrane staining) remains to be clarified.

The antigen recognized by 1A2 has not yet been identified; 1A2 may fail to react with the antigen due to denaturation of antigen under the experimental conditions used (such as detergent, heat, pH, etc.) When we attempted immunoprecipitation by using an S-methionine-labeled cell extract, we were unable to identify the protein recognized by 1A2. Moreover, for glycolipid antigens, an approach other than SDS gel electrophoresis may have to be used.

One unexpected finding of this study is that although 2H9 does not react with most B or T cell lymphomas or cell lines, it does react with some virus-transformed cell lines, such as HUT-102 (HTLV-I +), RPMI 8057, Daudi, and P3HR-1 (EBV+). The reason for this reactivity is not known. It should be noted that HeFi-1, an H-RS cell-specific antibody, also stains EBV-transformed B lymphoblastoid cell lines and HUT-102.[23] However, since these virus-positive T/B cell lines consistently express B or T cell markers, their cross-reactivity with 2H9 and HeFi-1 does not pose any diagnostic problems. We would like to emphasize that the 2H9-positive H-RS or THL cells also express other histiocyte or IR cell markers (ie, 1E9, Leu M1) and never express B or T cell antigens, even when these cells are induced with phorbol ester.[3] The 2H9 (mol wt 110 kd) and HeFi-1 (mol wt 120 kd) determinants do not seem to be identical, since only the 2H9 determinant can be modulated in the presence of antibody (Hsu et al, unpublished data).

In conclusion, we have obtained MoAbs that can be used for the differential diagnosis of histiocyte or IR-cell-related lymphomas. Previously, various diagnostic tests (ie, enzyme cytochemistry, detection of Fc or complement receptors, detection of lysosomal enzymes, and phagocytic assays) have been used for the diagnosis of THLs.[1,2,24] However, none of these methods has proved satisfactory because of lack of specificity and sensitivity. Although it is generally recognized that histiocytes and monocytes are related, the currently available monocyte-specific MoAbs, such as OK M1, Mo2, Leu M2, and Leu M3 do not react with the neoplastic cells of THL, and they cannot distinguish THL from other large cell lymphomas.[25] The three MoAbs (2H9, 1E9, and 1A2) that we have described appear to be useful not only for the diagnosis of THL but also for malignant histiocytosis and HD. Because these MoAbs react extremely rarely with the normal cell membranes of lymphoid or nonlymphoid cells, it may be possible to use them as effective agents for therapy or immunoimaging in the near future.

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Primary Systemic Amyloidosis: Multivariate Analysis for Prognostic Factors in 168 Cases

By Robert A. Kyle, Philip R. Greipp, and W. Michael O'Fallon

One hundred sixty-eight patients with primary systemic amyloidosis (AL) were identified. Median survival after diagnosis was 12 months and ranged from 4 months for patients presenting with congestive heart failure to 50 months for those presenting with peripheral neuropathy only. Utilizing the proportional-hazards model in a stepwise multivariate fashion to evaluate the simultaneous influence of putative risk factors as of diagnosis revealed that congestive heart failure, urine light chain, hepatomegaly, and multiple myeloma were the major factors adversely affecting survival during the first year after diagnosis. Serum creatinine, multiple myeloma, orthostatic hypotension, and monoclonal serum protein were the most important variables adversely affecting survival for patients surviving 1 year. These models were used to categorize patients according to the variables in the models into low-, moderate-, and high-risk groups for the first year after diagnosis and separately for subsequent years. The influence of these variables on survival is important in stratification of patients randomized to prospective clinical trials.

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ALTHOUGH amyloidosis has been recognized for more than a century, there has been no analysis of prognostic factors. Amyloid, a substance that appears to be homogeneous and amorphous under the light microscope, consists of rigid, linear, nonbranching aggregated fibrils. The fibrils consist of the variable portion of a monoclonal light chain, are arranged in a β-pleated sheet formation, are insoluble, generally resist proteolytic digestion, and constitute the amyloid deposits that replace and destroy normal tissues. Except for multiple myeloma, which is seen in about one-fifth of the patients with primary amyloidosis (AL), patients have no evidence of preceding or coexisting disease responsible for the amyloidosis.

In two series of patients with primary amyloidosis (AL), the median survival after histologic diagnosis of amyloid was approximately 1 year.[1,2] This short survival and the lack of an analysis of prognostic factors in primary amyloidosis (AL) prompted us to undertake this study.

MATERIALS AND METHODS

All Mayo Clinic records reporting a histologic diagnosis of amyloidosis from Jan 1, 1970, to Dec 31, 1980, were reviewed, and the data were abstracted on sheets suitable for keypunching. All laboratory data were obtained from Mayo Clinic records within 1 month of the histologic diagnosis of amyloidosis. All parameters were measured in all patients. The data for multiple visits were recorded on separate sheets, and a longitudinal view was obtained. Patients with secondary amyloidosis or with localized, familial, or senile amyloidosis were excluded. Patients with amyloidosis and multiple myeloma were included because dfferentiation on this basis is often difficult inasmuch as these conditions represent the same fundamental process. In a recent report,[3] the presence or absence of myeloma in patients with amyloidosis did not influence survival. All patients were followed up until death or for at least 1 year after the diagnosis of amyloidosis.

Standard univariate statistical methods were used to summarize the distribution of each of the parameters. Survival curves were estimated by the Kaplan-Meier technique.[4] The proportional-hazards method of Cox was used to determine the variables that influenced survival separately for the first year after diagnosis and for subsequent years.[5]

RESULTS

One hundred sixty-eight patients with primary systemic amyloidosis (including 40 with multiple myeloma) were identified (Table 1). Amyloidosis was diagnosed on the basis of finding amyloid deposits in tissue by the use of appropriate staining procedures or of demonstrating amyloid fibrils with electron microscopy. Seventy percent of the patients had a positive rectal biopsy. Biopsies of the kidney, small intestine, and sural nerve were positive in 83% to 100% of specimens studied.

Loss of weight was noted in 42% of the patients; the amount of the loss contributed significantly to the prediction of early survival. Among those who lost weight, the loss ranged from 2.3 to 40.9 kg (5 to 90 lb), with a median of 9.1 kg (20 lb). Fatigue was common, being found in 55% of the 168 patients. Purpura was found in 15% and gross bleeding in 9%. Some patients had more than one symptom.

The liver was palpable in one-third of the patients but extended more than 10 cm below the right costal margin in only two. The spleen was palpable in only 4% and was modest in size; none extended >3 cm below the left costal margin. Macroglossia, present in one-fourth of the patients, occasionally constituted the major manifestation of the disease. Lymphadenopathy was infrequent.

The syndromes present at the time primary systemic amyloidosis was diagnosed are listed in Table 2. Nine patients (5.3%) had a malabsorption syndrome. Symptoms of nephrotic syndrome and congestive heart failure existed approximately 1 to 3 months before the diagnosis of amyloidosis, while symptoms of peripheral neuropathy or carpal tunnel syndrome were present for approximately a year before amyloidosis was diagnosed. Congestive heart failure and orthostatic hypotension frequently developed during the patient's illness.

Laboratory studies. Anemia was not a prominent finding, the initial median hemoglobin value being 12.7 g/dL. Of

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Table 1. Age and Sex Distribution of 168 Patients With Primary Systemic Amyloidosis (AL)

Age (yr)	Males	Females	Total No.	Total %
<40	2	0	2	1
40-49	11	6	17	10
50-59	29	15	44	26
60-69	30	25	55	33
70-79	31	13	44	26
≥80	6	0	6	4
Total*	109 (65%)	59 (35%)	168	100

*Median, 64 years.

the 18 patients with an initial level <10 g/dL, 8 had multiple myeloma, 8 had renal insufficiency, 1 had gastrointestinal bleeding, and 1 had no apparent cause. Leukopenia was found in only 1%, thrombocytopenia in 1%, and thrombocytosis (> 500,000/mm^3) in 7%. Renal insufficiency was evident initially in 55% of the males and 59% of the females. Twenty-six percent of the males and 12% of the females had an initial creatinine level > 2.0 mg/dL.

Serum protein findings. More than half of the patients had either hypogammaglobulinemia or a normal serum protein electrophoretic pattern. A localized band or spike was found in 74 patients, and when present, it was usually of only modest size (median, 1.3 g/dL). Immunoelectrophoresis revealed a monoclonal serum protein in 69%. Unexpectedly, 26% of the patients had a free monoclonal light chain in the serum (Bence Jones proteinemia). Lambda light chains predominated (λ in 83 and κ in 31 cases).

Urine protein findings. Proteinuria (routine urinalysis) was found in 80% of the patients. Electrophoresis of concentrated urine revealed a globulin band in two-thirds of the patients and an albumin peak in more than 70%.

Immunoelectrophoresis of an adequately concentrated urine specimen showed a monoclonal light chain in 75% of the patients during their disease. In two-thirds of the patients, the light chains were λ. A monoclonal protein was found in the serum or urine in 89% of the patients.

Other studies. Bone marrow plasma cell counts ranged from 1% to 99%, with a median of 9%. Roentgenograms were normal in 62% of the patients. Osteolytic lesions were found in 15 patients; all had overt multiple myeloma.

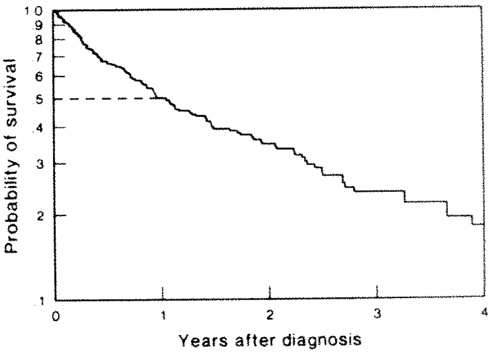

Fig 1. Probability of survival after diagnosis of primary systemic amyloidosis (AL).

Therapy and cause of death. Eighty-four percent of the patients received an alkylating agent some time during their illness. Colchicine was given to 13 patients. Nineteen patients did not receive any therapy. Cardiac involvement accounted for 40% of the deaths, but the percentage was probably higher because many patients who died of "amyloidosis" may have died of cardiac disease. Only 1 patient died of another malignancy—malignant melanoma.

Survival by syndromes. Median survival of the 168 patients after the diagnosis of amyloidosis was 12 months (Fig 1). All patients were followed up, if alive, for at least 1 year, and, as of the last follow-up, 79% had died. In contrast, the estimated median survival from diagnosis of amyloidosis to death for patients presenting with various syndromes ranged from 4 months for those presenting with congestive heart failure to 50 months for those presenting only with peripheral neuropathy (Table 3). Death of patients presenting with only the carpal tunnel syndrome or peripheral neuropathy (or both) was due to eventual involvement of other organs.

Seventeen of the 54 patients presenting with a nephrotic syndrome also had either congestive heart failure or orthostatic hypotension at presentation. Nine of the 54 survived more than 3 years after the diagnosis of amyloidosis, and only 2 of them are now known to have died, 1 of malignant

Table 2. Syndromes at Time of Diagnosis of Primary Systemic Amyloidosis (AL)*

Condition	At or Before Diagnosis of Amyloidosis No.	At or Before Diagnosis of Amyloidosis %	Month Before Diagnosis of Amyloidosis Median	Month Before Diagnosis of Amyloidosis Range	No. After Diagnosis of Amyloidosis	Total No.	Total %
Congestive heart failure	38	23	3.5	0-12	19	57	34
Nephrotic syndrome	54	32	1.5	0-36	6	60	36
Carpal tunnel syndrome	46	27	9.2	0-227	1	47	28
Orthostatic hypotension	23	14	6.2	0-39	9	32	19
Peripheral neuropathy	28	17	12.8	0-58	1	29	17

*Some patients had more than one condition.

Table 3. Survival by Presenting Symptoms of 168 Patients With Primary Systemic Amyloidosis (AL)

	Patients	Median Survival (mo)
Total group	168	12
Hierarchical groups*		
Congestive heart failure	38	4
Orthostatic hypotension	17	12
Nephrotic syndrome	37	16
Carpal tunnel syndrome	28	23
Peripheral neuropathy	11	50

*Patients were placed only in the first group they occurred in. Thus, none of the 37 patients listed as presenting with a nephrotic syndrome had either congestive heart failure or orthostatic hypotension, but some may have had the carpal tunnel syndrome or peripheral neuropathy.

Table 5. A Model Involving Five Variables Related to Survival During First Year After Diagnosis of Primary Systemic Amyloidosis (AL)

Variable	Coefficient	*P* Value
Congestive heart failure (CHF)	1.43	<0.0001
Urine light chain (ULC)	0.97	0.0064
Hepatomegaly (HEP)	0.58	0.0106
Multiple myeloma (MM)	0.65	0.0126
Monoclonal serum protein (MCS)	−0.87	0.0008

melanoma and 1 of suicide. One patient continues on dialysis more than 12 years after diagnosis. Only 3 patients who had congestive heart failure at presentation survived more than 2 years.

Analysis of survival. Because the assumption of proportional-hazards functions was not satisfied over the entire follow-up period but did hold separately within the first year and subsequently, the proportional-hazards method of Cox was used separately for these two periods in an attempt to evaluate the influence of the variables on survival. Coincidentally, half of our patients were dead at 1 year.

Analysis of survival during the first year. Four variables—congestive heart failure (CHF), urinary light chain (ULC), hepatomegaly, and amount of weight loss—had a highly significant influence on survival during the first year (Table 4). Four other variables (albuminuria, elevated serum creatinine, sex, and myeloma) were significant ($P < 0.05$).

The proportional-hazards model was then employed in a step-wise multivariate fashion to evaluate the simultaneous influence of combinations of the variables. The first three variables that were entered into the model, and their *P* values on entry, were CHF ($P < 0.0001$), ULC ($P = 0.0001$), and MCS (monoclonal serum protein) ($P = 0.009$).

When the step-wise process was allowed to continue without concern for the implications of the limited data available in some subsets of patients, hepatomegaly and multiple myeloma were identified as having an additional effect on survival ($P < 0.05$) after the effects of the first three variables had been accounted for.

The possibility that some of these five variables interact with each other in their influence on survival cannot be denied. However, the numbers of patients with combinations of variables is too small to address the question of such potential interactions in a satisfactory manner. The model (Table 5) summarizing the influence of the five variables assumes no interactions among them, and therefore the joint influences predicted by the model are based on the distribution of the individual variables, and predictions are possible, even when data are sparse.

The coefficients of the variables in this model suggest that a diagnosis of CHF is highly detrimental to survival, the presence of ULC somewhat less detrimental, the presence of HEP and MM about equal and even less detrimental, and the presence of MCS actually "protective." This model was used to create a gradient of risk, and three classes of patients were defined according to this gradient. These classes, referred to as low, moderate, and high risk, are defined as (1) low risk: Patients with no factors or MCS alone or MCS and one of HEP, MM, ULC. (2) Moderate risk: Patients with one of HEP, MM, ULC or MCS and two of HEP, MM, ULC or MCS and CHF. (3) High risk: Patients with CHF alone or all factors except CHF or CHF with or without MCS with one or more of HEP, MM, ULC or two or more of HEP, MM, ULC.

When the 168 patients were classified according to these definitions, there were 60 patients in the high-risk group, 70 in the low-risk group, and 48 in the moderate-risk group. The Kaplan-Meier estimates of the survival curves for these three

Table 4. Effect of Individual Variables on Survival Curves

During First Year		After First Year	
Variable	*P* (log-rank)	Variable	*P* (log-rank)
Congestive heart failure	.0001	Hemoglobin	.0022
Urinary light chain	.0001	Serum creatinine	.0023
Hepatomegaly	.0002	Myeloma	.0037
Weight loss (amount)	.0055	Monoclonal serum protein	.0370
Electrophoretic pattern of albumin in urine	.0108	Urinary light chain	.0540
Elevated serum creatinine (>2.0 mg/dL)	.0270		
Sex	.0320		
Myeloma	.0420		

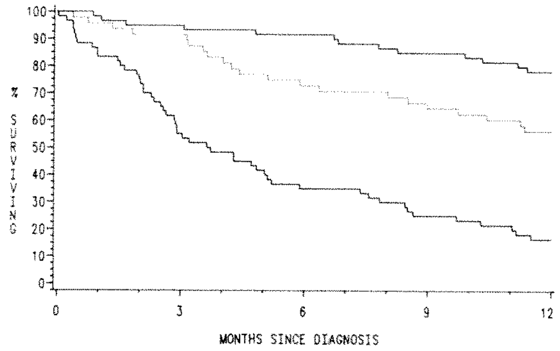

Fig 2. Kaplan-Meier estimates of the survival curves for low-, moderate-, and high-risk patients with primary systemic amyloidosis (AL).

classes are illustrated in Fig 2. The three curves are significantly different ($P < 0.0001$, log-rank test) and demonstrate a clear classification of the patients into three groups according to likelihood of surviving 1 year after a diagnosis of amyloidosis.

Analysis of survival after 1 year. The proportional-hazards model was also used to identify variables whose value at diagnosis influenced the rate of subsequent mortality among the 84 patients who survived 1 year after diagnosis (Table 4). Hemoglobin level, serum creatinine level, and presence of multiple myeloma have a highly significant influence on survival ($P < 0.005$), with MCS and ULC being of borderline significance.

As before, the proportional-hazards model was used in a step-wise multivariate fashion to evaluate the simultaneous influence of several variables on survival and/or to evaluate the influence of a variable after the influence of other variables has been taken into account.

In the modeling process, hemoglobin and serum creatinine levels appeared to be surrogates of each other, and the final model ($P < 0.0001$) included serum creatinine, multiple myeloma, orthostatic hypotension, and monoclonal protein in the serum (Table 6). Although no interactions were found to be significant, the sample size limits our ability to detect them. As before (in reference to year 1), the model is used to establish a gradient of risk, and three classes of patients are defined on the basis of the values of the four variables at the time of the diagnosis of amyloid and referred to as low, moderate, and high risk. These classes are (1) low risk: Serum creatinine ≤ 2.0 and no risk factors (MM, OH, MCS); (2) moderate risk: Serum creatinine 2.0 to 6.5 and no risk factors or serum creatinine < 2.0 and either MM or MCS; (3) high risk: Serum creatinine > 6.5 and no risk factors or serum creatinine > 2.0 and either MM or MCS or OH alone or two or more of MM, MCS, OH.

Of the 84 patients who survived 1 year after the diagnosis of amyloid, 14 were in the low-risk group, 38 in the moderate-risk group, and 32 in the high-risk group. Figure 3 illustrates the estimated survival curves (Kaplan-Meier) for these three groups. The curves differ significantly ($P < 0.0001$, log-rank test).

DISCUSSION

In three series comprising almost 500 cases of primary systemic amyloidosis (AL) from the Mayo Clinic, the median survival was 14.7 months or less.[1,2,6] Daniels and Hewlett[7] reported that the median survival of patients with primary amyloidosis was 5 months, while in another series,[8] no patients with primary amyloidosis (AL) survived more than 12 months after diagnosis. In a series of 32 patients with primary systemic amyloidosis with or without myeloma, the median survival was 14 months.[3] In 1976, Pruzanski and Katz[9] reported a mean survival of 28 months for 26 patients with primary amyloidosis who had died.

Table 6. Variables Affecting Survival After First Year Following the Elimination of Hemoglobin in Cases of Primary Systemic Amyloidosis (AL)

Variable	Coefficient	*P* Value
Serum creatinine	0.21	0.0001
Multiple myeloma (MM)	1.07	0.0014
Orthostatic hypotension (OH)	1.75	0.0007
Monoclonal serum protein (MCS)	1.04	0.0065

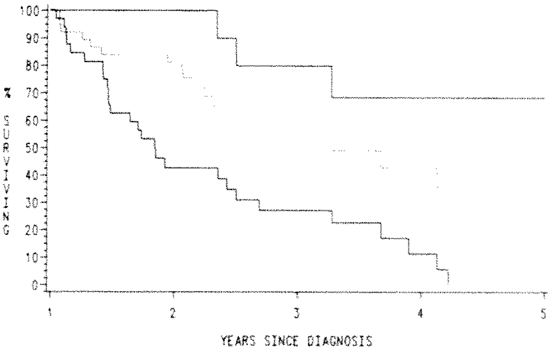

Fig 3. Kaplan-Meier estimates of the survival curves for low-, moderate-, and high-risk patients with primary systemic amyloidosis (AL) who survived 1 year after diagnosis.

The median survival from diagnosis of amyloidosis to death among the current series of 168 patients presenting with associated syndromes ranged from 4 months in those presenting with congestive heart failure to 50 months in those presenting only with peripheral neuropathy. It should be pointed out that the patients presenting only with peripheral neuropathy or the carpal tunnel syndrome who died usually did so from subsequent cardiac or renal involvement. In fact, cardiac involvement accounted for the deaths of 40% of the patients who died.

The median survival of the 168 patients in our series was 12 months. The major risk factor for survival during the first year was the presence of congestive heart failure. The presence of hepatomegaly, urinary light chain, and multiple myeloma also had an adverse effect on survival during the first 12 months. The presence of a monoclonal protein appeared to have a "protective" effect on survival but only during the first year. An explanation of this phenomenon is not readily available. Utilization of these variables allowed us to categorize the patients into low risk, moderate risk, or high risk the first year after diagnosis. Patients in the high-risk group had a median survival of 3.5 months, in contrast to more than 1 year for those in the low- or moderate-risk groups.

Utilization of the proportional-hazards model in a stepwise multivariate fashion revealed that after the first year, patients with more severe systemic amyloidosis had died and that serum creatinine levels, multiple myeloma, orthostatic hypotension, and monoclonal serum protein in the urine were the most important variables affecting survival. Patients in the high-risk group had a median survival of 10 months after the first year (total, 22 months), whereas the median survivals in the low- and moderate-risk groups exceeded 28 months after the first year (total, 40 months). It should be emphasized that the significant variables influencing survival during the first year after diagnosis—congestive heart failure, urinary light chain, hepatomegaly, and loss of weight—had a negligible role in affecting survival after the first year.

This study permits a more accurate assessment of survival at the time of diagnosis of primary systemic amyloidosis. The influence of these variables on survival is important in the stratification of patients randomized to prospective clinical trials.

ACKNOWLEDGMENT

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Functional Characterization of Platelet-Bound Factor XIa: Retention of Factor XIa Activity on the Platelet Surface

By Peter N. Walsh, Dipali Sinha, Alice Koshy, Frances S. Seaman, and Harlan Bradford

Previously we have shown that both factor XI and factor XIa are bound specifically to distinct, high-affinity sites on the surface of activated platelets in the presence of high Mr kininogen. To determine the functional significance of factor XIa binding to platelets, bound factor XIa has now been compared with the unbound enzyme. Platelets incubated with thrombin, high Mr kininogen, and ^{125}I-labeled factor XIa bound 130 to 500 molecules of factor XIa per platelet. Scatchard analysis of binding data give a dissociation constant (Kd) of 822 pmol/L ± 140 (SEM). Rates of factor IX activation, assayed by release of trichloroacetic acid-soluble ^{3}H-labeled activation peptide from purified [^{3}H]-factor IX, were similar when factor XIa was bound to platelets and when it was free in solution. The platelet-bound factor XIa was isolated by centrifugation through 20% sucrose and was functionally characterized both in a factor XIa coagulation assay and in the factor IX activation peptide release assay in comparison with unbound factor XIa in the presence of treated platelets. The functional activity of platelet-bound factor XIa as a factor IX activator as well as its structural integrity were shown to be fully retained on the platelet surface. Since platelets bind factor XI and promote its proteolytic activation to factor XIa, factor XIa binding to platelets may serve to localize factor IX activation to the hemostatic plug, where factor XIa is protected from inactivation by plasma protease inhibitors and where acceleration of subsequent coagulation reactions can occur.

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PLATELETS participate in blood coagulation at various stages of the intrinsic system by interacting specifically with coagulation proteins and promoting the activation of factor XII,[1] factor XI,[1] factor X,[2] and prothrombin.[3,4] Previously we have demonstrated that the zymogen factor XI can interact specifically with the surface of activated platelets in the presence of the protein cofactor, high Mr kininogen, with which it associates in plasma as a noncovalent complex.[5] A consequence of this high-affinity, specific, saturable binding of factor XI to platelets is the proteolytic activation of factor XI in the presence of kallikrein by both factor XII–dependent and factor XII–independent mechanisms.[1] We have also shown that the enzyme, factor XIa, binds specifically to a site on activated human platelets distinct from that for factor XI.[6] Since an analysis of the factor XIa catalyzed activation of factor IX suggests that this is a kinetically favorable reaction,[7] we have now examined the interaction of factor XIa with platelets to determine the functional consequences on factor IX activation.

METHODS

Materials. All chemicals were the best grade commercially available and were purchased from Sigma Chemical Co, St. Louis, Mo; Fisher Chemical Co, Fairlawn, NJ; or J.T. Baker Chemical Co, Phillipsburg, NJ. Plasmas deficient in coagulation factors were purchased from George King Biomedical, Overland Park, Ka. Betaphase scintillation fluid was obtained from West Chem Products, San Diego, Calif. Carrier-free Na^{125}I and tritiated sodium borohydride (75 Ci/mmol) were purchased in crystalline form in sealed ampoules from New England Nuclear, Boston, Mass. Iodogen (1,3,4,6-tetrachloro-3, 6-diphenylglycouril) was obtained from Pierce Chemical Co, Rockford, Ill. Ultrol Hepes (N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid) was from Calbiochem-Behring Corp, La Jolla, Calif, and Sepharose 2B was from Pharmacia Fine Chemicals, Piscataway, NJ. The chromogenic substrate Pyr-Gly-Pro-Arg-paranitroanilide 2 HCl (S-2366) was a kind gift from AB KABI Peptide Research, Molndal, Sweden. Methyl silicon oil (1.0 DC200) and Hi Phenyl silicon oil (125 DC 550) were obtained from William F. Nye, Inc, Fairhaven, Mass.

Preparation of platelet suspensions. Platelet-rich plasma was obtained from citrated human blood as previously described.[8] Platelets were separated from plasma by gel filtration[6,9] into calcium-free, Hepes-buffered Tyrode's solution of the following composition: NaCl, 126 mmol/L; KCl, 2.7 mmol/L; $MgCl_2$ $6H_2O$, 0.98 mmol/L; NaH_2PO_4 H_2O, 0.376 mmol/L; Hepes, 15 mmol/L; dextrose, 5.6 mmol/L; bovine serum albumin, 0.35%; pH 7.4. Alternatively, platelets were washed by the procedure of Mustard et al[10] and were finally suspended in calcium-free, Hepes-buffered Tyrode's solution. Platelets were counted electronically using a Model ZBI Particle Counter (Coulter Electronics, Inc, Hialeah, Fla).

Purification of proteins. Factor XI was purified from 2 L of human plasma by a modification[11] of the method of Bouma and Griffin[12] as previously described.[7] The purified protein appeared homogeneous on polyacrylamide gel electrophoresis in the presence of SDS as previously published,[7] and had a specific activity of 270 U/mg protein. It was stored in 0.2 mol/L sodium acetate, 0.6 mol/L NaCl, pH 5.3 at −70 °C. Human high Mr kininogen was purified to apparent homogeneity and specific activity of 15 U/mg, and was converted to the two-chain, kinin-free form as previously described by Kerbiriou and Griffin.[13] Human factor IX was purified to a specific activity of 225 U/mg by a modification[7] of methods described by DiScipio et al[14] and by Miletich et al[15] and was a single band on SDS gel electrophoresis as previously published.[7] It was stored in 0.012 mol/L Tris, 0.045 mol/L glycine, pH 8.3 at −185 °C. Bovine two-chain factor XIIa was kindly provided by Dr E.P. Kirby of the Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pa, as a homogenous protein that hydrolyzed 3.78 × 10^9 mol of the chromogenic substrate S2302/min/μg protein at pH 8.0 and 37 °C. Human alpha-thrombin was a gift from Dr J.W. Fenton, II, New York State Department of Health, Albany, NY.

Preparation and characterization of factor XIa. Purified factor XI was activated by incubation at 37 °C with bovine factor XIIa as

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previously described.[6] Maximal factor XIa coagulant activity appeared at two hours at which time 95% of the protein was present in cleavage products of Mr 48,000 and 32,000 as previously published.[6] The factor XIa was assayed for amidolytic activity (127 U/mL), coagulant activity (135 U/mL), and by immunoradiometric assay (750 μg/mL) as previously described[16] and was thereby determined to have a specific activity of approximately 170 to 180 U/mg protein.

Radiolabeling of proteins. Factor XI was labeled with ^{125}I utilizing the Iodogen method.[17] Most of the free ^{125}I was removed from the labeled protein by passage over a 1 mL G-25 column[18] and the protein was then dialyzed further in the presence of ovalbumin (1 mg/mL) to remove residual ^{125}I. The factor XI was then converted to factor XIa as described above, was shown to retain >85% of its coagulant activity and had a specific radioactivity of 1117 CPM/ng protein. In binding assays, when the proportion of unlabeled to radiolabeled factor XIa was varied with a constant final concentration of total factor XIa, a linear relationship was observed between the percentage of ^{125}I-labeled factor XIa in the mixtures and the amount bound.[6] This indicates that the affinities of the radiolabeled and unlabeled factor XIa were comparable. Factor IX was labeled with tritium by a modification of the method described by Van Lenten and Ashwell[19] and adapted for bovine factor X by Silverberg et al[20] as previously described.[7] The labeled protein was finally purified by alkaline gel electrophoresis,[21] appeared homogeneous as judged by SDS gel electrophoresis and fluorography[22] as previously reported,[7] and >99% of the radioactivity was precipitable in 5% trichloroacetic acid. The tritiated factor IX had a specific radioactivity of 475 CPM/ng and retained >90% of its coagulant activity compared with unlabeled factor IX.

Coagulation assays. Factors IX and XI and high Mr kininogen were assayed utilizing minor modifications of the kaolin-activated partial thromboplastin time[23] using appropriate congenitally deficient substrate plasmas, and results were quantitated on double logarithmic plots of clotting times versus concentration of pooled normal plasma. Factor XIa was measured utilizing a similar assay carried out in polystyrene tubes without the addition of kaolin. The quantitation of factor XIa activity was accomplished by means of a standard curve of the logarithm of clotting time versus the logarithm of the concentration of purified factor XIa.

Radiometric assay of factor IX activation. The release of trichloroacetic acid (TCA)–soluble ^{3}H-labeled activation peptide from (sialyl-^{3}H)-factor IX was measured as previously described.[7] Briefly, assays were carried out in Tris (50 mmol/L), NaCl (100 mmol/L), pH 7.5 (Tris buffered saline or TBS), containing bovine serum albumin (1 mg/mL) in reaction volumes of 300 to 500 μL. At given times of incubation of factor XIa with (sialyl-^{3}H)-factor IX in the presence of 5 mmol/L $CaCl_2$ at 37 °C, 80 μL samples were removed and added to 240 μL of an ice-cold mixture containing one part TBS and two parts 50 mmol/L EDTA, pH 7.5. To this mixture was added 160 μL of ice-cold 15% TCA or TBS, and following vigorous vortexing for 2 minutes it was centrifuged at 10,000 g for 3 minutes in a bench top Brinkman Model 3200 Microfuge (Brinkman Instruments, Inc, Westbury, NY). Aliquots (100 μL) of the supernatants were removed into 10 mL of scintillation fluid and counted in triplicate in a Beckman LS 8000 scintillation counter (Beckman Instruments, Inc, Fullerton, Calif).

Binding studies. Determinations of binding of factor XIa to platelets were carried out as previously described.[6] Briefly, 100 μL of gel-filtered platelets (2 to 4 × 10^8/mL) in calcium-free, Hepes-buffered Tyrode's solution, pH 7.4, were incubated at 37 °C with reactants including ^{125}I-labeled factor XIa in a total volume of 115 μL. All incubations were carried out in duplicate, and results represent means of two determinations, which were always within 5% of one another. After specified incubation times, 100 μL aliquots were removed and centrifuged in a microfuge (Model B, Beckman Instruments, Inc, Cedar Grove, NJ) through a mixture of silicone oils (5 vol. DC550:1 vol DC 200) in microsediment tubes with narrow bore extended tips (Sarstedt, Inc, Princeton, NJ). The tips containing the sediments were amputated with wire cutters and the sediments and supernatants counted separately in a gamma counter (Model CG 4000; Intertechnique, Plaisir, France).

Protein analyses. Protein assays were carried out as described by Lowry et al[24] and were also determined by absorbance at 280 nm employing $E^{1\%}_{280}$ = 13.4 for factor XI.[25] and 7.01 for high Mr kininogen.[26] Polyacrylamide slab gel electrophoresis in SDS was done by the procedure of Laemmli.[27]

RESULTS

Binding of factor XIa to platelets. Previously we have demonstrated that factor XIa binds specifically to a site on activated human platelets distinct from that for factor XI. The requirements for binding include the presence of high Mr kininogen and a platelet agonist, such as thrombin. A series of five identical experiments was carried out to determine the number and affinity of specific binding sites for factor XIa on activated human platelets obtained from five separate donors (Figs 1 and 2). In these experiments, gel-filtered platelets were incubated with high Mr kininogen and thrombin in the presence of ^{125}I-labeled factor XIa added at various concentrations, and total binding was determined. Nonspecific binding was also measured at two or three concentrations of ^{125}I-labeled factor XIa in the presence of a 50-fold excess of unlabeled factor XIa compared with the highest concentration of ^{125}I-labeled factor XIa and was found to be nonsaturable; ie, it was a linear function of added ligand, and ranged between 10% and 20% of total binding. Specific binding was determined as the difference between total and nonspecific binding after a 4-minute incubation at

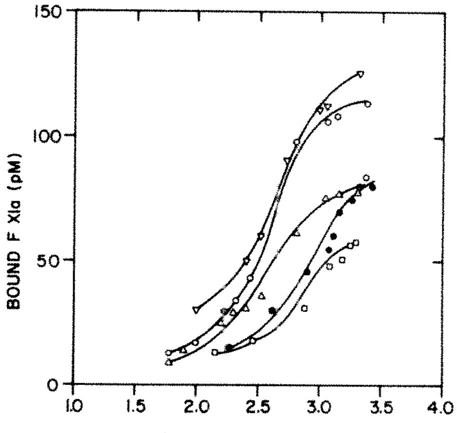

Fig 1. Specific binding of ^{125}I-factor XIa to platelets as a function of factor XIa concentration. Gel-filtered platelets at concentrations of 1.8 to 4.3 × 10^8/mL were incubated at 37 °C for 4 minutes within thrombin (0.25 U/mL), high Mr kininogen (12.5 μg/mL), and various concentrations of factor XIa. Nonspecific binding was measured in the presence of 14 μg/mL of unlabeled factor XIa and was subtracted from total binding to give specific binding. Results obtained in duplicate in five separate experiments with five separate donors are plotted as recommended by Klotz.[29]

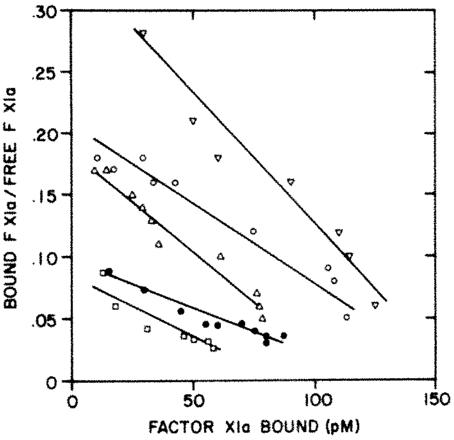

Fig 2. Scatchard plot of data presented in Fig 1.

which time it was demonstrated that specific binding had achieved a plateau and was more than 80% reversible.

To determine whether saturation had indeed been achieved under these experimental conditions, specific binding data for 5 separate experiments were plotted as recommended by Klotz[28] in Fig 1. This method of plotting ligand binding data has been suggested as means to determine whether or not a sufficient amount of ligand has been added in a binding experiment to approach saturation of binding sites. Thus, when the data are plotted as the amount bound versus the logarithm of the amount of ligand added, the result should describe a sigmoid curve, with the inflection point representing half-maximal binding, if saturation of binding sites has, in fact, been approached. The data plotted in Fig 1 suggests that approximately half the points represent data obtained at concentrations greater than those required to achieve half-saturation, and therefore should justify Scatchard analysis[29] provided equilibrium between bound and free ligand can be demonstrated.

The same data depicted in Fig 1 were then replotted according to the method of Scatchard[29] in Fig 2 to obtain estimates of the number and affinity of binding sites. The data suggest the presence of a single class of binding sites since a straight line can apparently be drawn through each set of binding data. Dissociation constants (Kd) calculated for each experiment gave a mean value of 822 pmol/L (range, 471 to 1250; SD, 312; SEM, 140) and the mean number of binding sites per platelet was 291 (range 196 to 489; SD, 137; SEM, 61).

Activation of factor IX by platelet-bound factor XIa. To examine directly the functional consequences of the binding of factor XIa to platelets, platelets with the bound enzyme were compared with factor XIa in solution in the presence of platelets under conditions not promoting factor XIa binding (Fig 3). Since binding of factor XIa to platelets requires platelet activation and the presence of the protein cofactor, high Mr kininogen,[6] platelets were incubated with factor XIa, high Mr kininogen, and thrombin to allow equilibrium binding of factor XIa to platelets. The coagulant activity of platelet-bound factor XIa was compared with that of unbound factor XIa in the presence of platelets previously treated with thrombin and high Mr kininogen. Gel filtered platelets were incubated with high Mr kininogen and thrombin in the presence or absence of ^{125}I-labeled factor XIa. Both samples were centrifuged through 20% sucrose, the platelets resuspended, and the amount of factor XIa bound was determined by the number of counts recovered in the pellet of the sample to which ^{125}I-factor XIa had been added. A control experiment carried out in the presence of a 50-fold excess of unlabeled factor XIa indicated that 80% of the binding was specific, whereas 20% was nonspecific. It was then confirmed by centrifugation through silicone oil that 65% of the factor XIa sedimented with platelets through sucrose remained bound to the platelets after resuspension. This sample was assayed for factor XIa activity in factor XI–deficient plasma without added kaolin. The clotting times obtained at three dilutions are plotted in Fig 3 *v* the amount of factor XIa determined by gamma counting to be present in the samples. To the other sample of platelets treated with high Mr kininogen and thrombin and centrifuged through sucrose was added ^{125}I-labeled factor XIa at the concentration (determined by gamma counting) depicted in Fig 3. It was confirmed by centrifugation of this sample through silicone oil that less than 4% of the added factor XIa was bound to the platelets. Since the clotting times were similar in the sample with mainly (65%) bound factor XIa and in the sample with mainly (96%) free factor XIa, it is concluded that the coagulant activity of factor XIa is fully retained (neither enhanced nor inhibited) when it is bound to the platelet surface.

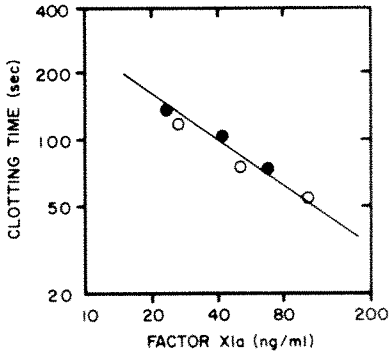

Fig 3. Factor XIa coagulant activity of platelet-bound factor XIa and unbound factor XIa in the presence of treated platelets. Gel-filtered platelets (2.0 × 10^8/mL) were incubated at 37 °C for 10 minutes in a total volume of 822 μL with high Mr kininogen (20 μg/mL) and thrombin (0.25 U/mL) in the presence or absence of ^{125}I-labeled factor XIa (3 μg/mL). The two samples were then centrifuged through 20% sucrose at 10,000 × g for 4 minutes and the pellets resuspended in 50 μL of Hepes-buffered Tyrode's solution with BSA (1 mg/mL) containing 50 μmol/L EDTA. The sample containing ^{125}I-labeled factor XIa (●) was counted in a gamma counter, found to contain the amount of factor XIa indicated, and was assayed at three dilutions for factor XIa coagulant activity as indicated in Methods. A similar amount of ^{125}I-factor Xa was added to the sample of treated platelets (○) which was also assayed for factor XIa coagulant activity.

The experiment presented in Fig 4 confirms this conclusion utilizing the radiopeptide release assay for factor IX activation. Gel-filtered platelets (4.5 × 10^8/mL) were incubated with high Mr kininogen (20 μg/mL) and thrombin (0.25 U/mL) in the presence of ^{125}I-labeled factor XIa (2.8

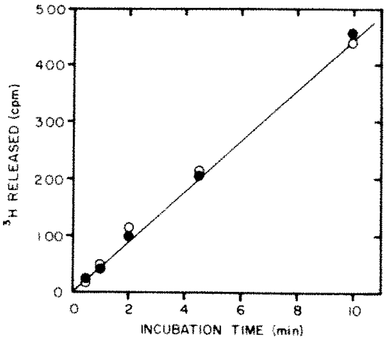

Fig 4. Effect of platelet-bound *v* free factor XIa on factor IX activation. Gel-filtered platelets (4.5 × 10^8/mL) were incubated for 8 minutes at 37 °C with high Mr kininogen (20 μg/mL), thrombin (0.25 U/mL), and factor XIa (2.8 μg/mL) or buffer. The two samples were then centrifuged through 20% sucrose and the pellets resuspended in 1 mL of calcium-free, Hepes-buffered Tyrode's solution. The amount of factor XIa bound to the platelets in the first sample (●) was determined as indicated in Results to be 132 ng/mL (8.25 × 10^{-10} mol/L), and an equivalent amount of factor XIa was added to the treated platelet sample (○). To each sample was added [sialyl-^{3}H] factor XI (2 μg/mL or 3.5 × 10^{-8} mol/L) and $CaCl_2$ (5 mmol/L). After the incubation times indicated, aliquots of the incubation mixtures were sampled for measurement of TCA-soluble ^{3}H release as indicated in Methods.

μg/mL). After an eight-minute incubation at 37 °C to allow binding of factor XIa to platelets, the amount bound was determined, after centrifugation through 20% sucrose, to be 4.7% of the amount added, ie, 132 ng/mL (8.25 × 10^{-10} mol/L) or 29 ng/10^8 platelets, of which 78% was specifically bound; ie, 22% of the binding was nonspecific as shown by a control experiment with a 50-fold excess of unlabeled factor XIa. Then, in a separate tube, unlabeled factor XIa or buffer was incubated with platelets, high Mr kininogen, and thrombin as indicated in the legend to Fig 4, the platelets sedimented through 20% sucrose and resuspended in Hepes-buffered Tyrodes solution. To the sample of platelets treated without factor XIa was added an amount of factor XIa equivalent to that bound to the platelets treated with factor XIa, ie, 132 ng/mL. It was confirmed in a separate incubation that 72% of the bound factor XIa remained bound whereas only 3.5% of the factor XIa was bound to the treated platelets. To the two samples containing mainly bound factor XIa and mainly unbound factor XIa, [sialyl-^{3}H]-factor IX and $CaCl_2$ were added for determination of factor IX activation by the peptide release assay. The results (Fig 4) confirm those of the experiment in Fig 3 and demonstrate that the activity of factor XIa as a factor IX activator is fully retained (neither enhanced nor inhibited) when it is bound to the platelet surface.

DISCUSSION

The experiments reported here confirm and extend our previous observation[6] that activated platelets interact specifically with the enzyme, factor XIa, which binds reversibly to high affinity sites distinct from those for the zymogen, factor XI.[5] A cofactor required for the binding of factor XIa to platelets is high Mr kininogen, which is also required for factor XI binding to platelets.[5,6] However, whereas both factor XI and high Mr kininogen are bound to activated platelets in the presence of physiologic concentrations of Zn^{2+} (25 to 50 μmol/L) and Ca^{2+} (2 mmol/L) ions,[32] factor XIa binding occurs in the absence of Zn^{2+} and Ca^{2+} ions or in the presence of EDTA, conditions which preclude the binding of high Mr kininogen.[6] Therefore the mechanism by which high Mr kininogen promotes factor XIa binding to activated platelets in the absence of Zn^{2+} and Ca^{2+} ions is not likely to involve coordinate binding of high Mr kininogen with factor XIa. This suggests that, whereas factor XI and high Mr kininogen may bind to platelets as a complex, similar to the coordinate binding to platelets of the complex of factor Xa and factor Va,[4] the platelet binding site for factor XIa is not likely to be plasma high Mr kininogen. The possible role in factor XIa binding of platelet high Mr kininogen, which appears to be stored in and secreted from platelet alpha-granules,[33] remains to be investigated.

Specific binding at saturation of factor XIa to thrombin-activated platelets in the presence of high Mr kininogen varied over a 3.7-fold range when platelets from five separate donors were employed (Figs 1 and 2). That this binding was, in fact, saturable is substantiated by analysis of the binding data according to the method of Klotz,[28] as shown in Fig 1. The partially dissociable nature of binding at later times of incubation of factor XIa with platelets initially[6] made us reluctant to derive binding constants by Scatchard analysis.[29] However, we have now carried out binding measurements at early time points when the majority of factor XIa binding is reversible, and subjected the data to Scatchard analysis, assuming equilibrium between free and bound ligand (Fig 2). The derived binding constants are in general agreement with our previously published conclusions[6] and indicate the presence of 130 to 500 factor XIa binding sites per platelet with a mean of 291 sites per platelet and a dissociation constant (Kd) of 822 pmol/L ± 140 (SEM).

The primary focus of the present study is to characterize the bound factor XIa functionally, having previously characterized it structurally and found it to be indistinguishable from unbound factor XIa; ie, a 160,000 Mr protein by SDS gel electrophoresis, consisting of a 50,000 Mr heavy chain and a 30,000 Mr light chain on reduced gels.[6] These data provided evidence that the bound protein was factor XIa and not a contaminant and no evidence for high molecular weight covalent complex formation with a platelet membrane component or for proteolytic degradation of the bound ligand. To study the coagulant activity of the platelet-bound ligand, we have employed an assay procedure and an experimental protocol that permit a direct and unequivocal functional characterization of factor XIa bound to the platelet surface. The activation peptide release assay for factor IX activation is highly specific since it monitors a physical property of the molecule that correlates well with the development of factor IXa coagulant activity and with the enzymatic activity of added factor XIa measured either by chromogenic or coagulation assays.[7] Using this method to assay factor XIa we have shown in experiments not reported here that rates of factor IX activation are similar in the presence and absence of platelets and whether factor XIa is bound to platelets or not. Since less than 10% of the total amount of added factor XIa is bound to platelets in the presence of thrombin and high Mr kininogen at saturating concentrations of added ligand, it was important to measure factor IX activation by bound and by free factor XIa. We therefore isolated the platelet-factor XIa complex by centrifugation through 20% sucrose and demonstrated using a coagulation assay (Fig 3) and the activation peptide release assay (Fig 4) that the functional activity of platelet-bound factor XIa is identical to that of unbound factor XIa. In these experiments it was demonstrated that approximately 80% of the platelet-bound factor XIa was specifically bound. The present observations allow us to conclude that both the functional activity and the structural integrity of factor XIa are fully retained on the platelet surface under our experimental conditions.

It has previously been demonstrated that phospholipids can accelerate the rate of bovine factor IX activation by surface-bound factor XIa.[34] In these experiments factor IX activation was measured by a factor IXa clotting assay and by proteolytic cleavage of factor IX in glass tubes containing surface-bound factor XIa. Rates of activation were increased 2- to 3-fold in the presence of phospholipids. The relationship between these findings and our present results is not clear. It should be emphasized that our studies were carried out with highly purified coagulation proteins. It is possible that a cofactor present in normal plasma cound influence rates of factor IX activation by factor XIa, but to our knowledge no direct evidence to support such a possibility is available.

Previously we have reported experiments, utilizing crude preparations containing factor XIa activity and plasma protease inhibitor activity, that platelets appear to protect factor XIa from inactivation by proteinases.[2] We were therefore interested to determine, using highly purified proteins, whether platelet-bound factor XIa is protected from inactivation by alpha-1-protease inhibitor, which has been reported to account for 68% of the factor XIa inhibitory activity in human plasma.[35] The results of our experiments (manuscript in preparation) confirm the hypothesis that platelet-bound factor XIa is indeed protected from inactivation in the presence of alpha-1-protease inhibitor.

The interpretation of the present studies in the context of what is known about the role of platelets in coagulation reactions is a matter of conjecture. Platelets bind factor XI tightly and specifically[5] and accelerate the rate of factor XI activation in the presence of factor XIIa or kallikrein.[1] Factor XIa also binds tightly and specifically to a site on the activated platelet surface distinct from the binding site for factor XI.[6] These interactions therefore can be viewed as localizing the activation of factor XI and the activity of factor XIa to the hemostatic plug where coagulation enzymes including factor XIa and factor Xa are protected from inactivation by plasma proteinase inhibitors.[2,36,37] The present studies indicate that the functional activity and structural integrity[6] of factor XIa are fully retained when the protein is bound to platelets. Kinetic analysis of the factor-XIa–catalyzed activation of factor IX (Km = 0.49 μmol/L; kcat = 7.7/s) suggests that this is a physiologically significant reaction.[7] Since the present studies indicate that the reaction rates are similar when factor XIa is bound to platelets and when it is free in solution, we suggest that factor IX activation by platelet-bound factor XIa is also a kinetically favorable reaction. Furthermore the reaction may occur preferentially on the platelet surface where factor XI is bound and activated, where factor XIa is tightly bound and may saturate binding sites when 5% to 10% of the factor XI in plasma is converted to factor XIa,[6] and where factor XIa, as well as factor Xa, can be protected from inactivation by protease inhibitors. Thereafter rates of factor X activation[2] and prothrombin activation[3,4] are greatly accelerated by accessory factors on the platelet surface, consistent with the amplifier effect of coagulation reactions implicit in the cascade[38] or waterfall[39] theory of coagulation as originally presented.

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Effects of 2,3-Diphosphoglycerate on the Mechanical Properties of Erythrocyte Membrane

By Richard E. Waugh

Investigation by Schindler et al[1] and Sheetz and Casaly[2] have indicated that high (~10 mmol/L) concentrations of 2,3-diphosphoglycerate (2,3-DPG) have a destabilizing effect on erythrocyte membrane and the membrane skeleton. We have investigated changes in the membrane mechanical properties that occur at elevated 2,3-DPG levels in both intact cells and ghosts. The membrane shear modulus, viscoelastic recovery time constant, critical force, "plastic" viscosity, and material relaxation time constant were measured by standard micropipette and flow channel techniques. Intact cells showed no change in properties at physiologic ionic strength and 2,3-DPG concentrations of about 20 mmol/L, except for an increase in membrane viscosity resulting from an increased cellular hemoglobin concentration that occurs when the 2,3-DPG concentration is elevated. At ionic strengths 20% below physiologic and 2,3-DPG concentrations of ~20 mmol/L, decreases in membrane shear modulus and membrane viscosity were observed. In ghosts, no changes in these properties were observed at a 2,3-DPG concentration of 10 mmol/L and ionic strengths as low as 25% below physiologic, but a decrease in the force required to form tethers (critical force) was observed at physiologic ionic strength. The decrease in membrane shear modulus and viscosity of intact cells and the reduced critical force in ghosts are consistent with the results of other investigators. However, the difference in the effects of 2,3-DPG on ghosts and intact cells indicates that the effects of 2,3-DPG depend strongly on the conditions of the experiment. It appears unlikely that 2,3-DPG affects erythrocyte membrane material properties under physiologic conditions.

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THE erythrocyte membrane consists of a phospholipid bilayer containing many integral proteins, an extracellular glycocalyx, and a membrane-associated proteinaceous skeleton at the cytoplasmic surface of the bilayer. Mechanically, it is recognized that the bilayer behaves like a two-dimensional liquid. It resists changes in area elastically, but it can freely undergo constant area shear deformation (in-plane deformation), and particles in the bilayer are free to diffuse in the plane of the surface. The shear elasticity that the membrane exhibits is due to the presence of the membrane skeleton, which also acts to limit the rate at which some integral proteins diffuse in the surface. Recently, it was observed that the lateral mobility of integral membrane proteins was increased in erythrocyte ghosts containing elevated levels of 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP).[1] Subsequently, Sheetz and Casaly[2] observed that polyanions, notably 2,3-DPG and ATP, accelerated the rate at which isolated membrane skeletons dissociate in vitro. The dissociative effects of 2,3-DPG were found to be much stronger than the effect of ATP on the isolated skeleton, 2,3-DPG having a half maximal effect at 2.5 mmol/L and ATP having a half maximal effect at 8.0 mmol/L. These results supported the hypothesis that the increased mobility of integral proteins was due to a decrease in the stability of the cytoskeleton, or an increase in the rate of exchange of the associations among the membrane skeletal components. Because of the importance of the skeleton in maintaining proper mechanical function of the membrane, it was postulated that 2,3-DPG might also affect the mechanical properties of the membrane. This postulate was supported by the observations of Chasis et al.[3] that ghosts with elevated 2,3-DPG concentrations fragmented more readily than control ghosts in the ektacytometer. It was suggested that these effects might serve an important physiologic function.

To test the effects of elevated concentrations of 2,3-DPG on the mechanical properties of erythrocyte membrane we have measured the intrinsic membrane material constants that have been defined to characterize the membrane mechanical behavior. We have performed these measurements on the membranes of both intact cells and resealed erythrocyte ghosts. The particular material constants that were measured are described and defined in the following section.

Definition of Material Properties

The mechanical behavior of the red cell membrane is complex. (For reviews see Evans and Hochmuth[4] and Evans and Skalak.[5]) Different constitutive relationships have been used to describe the material behavior, depending on the magnitude and duration of the applied forces. The resistance of the membrane to area changes is large, and the area of the membrane of each cell can be presumed to be constant during these experiments. The membrane resists in-plane extensions elastically. The maximum shear resultant in the surface, T_s, is related to the material extension ratio, λ, by the surface elastic shear modulus, μ[4]:

$$T_s = \frac{\mu}{2}(\lambda^2 - \lambda^{-2}) \qquad (1)$$

When surface deformations are dynamic, the membrane exhibits viscous character. For rapid deformations within the elastic regime, the total shear resultant can be written as the

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sum of the elastic and viscous contributions[4]:

$$\mathrm{T}_s = \frac{\mu}{2}(\lambda^2 - \lambda^{-2}) + 2\eta\,\mathrm{V}_s \qquad (2)$$

where η is defined as the membrane viscosity coefficient for rapid deformation and V_s is the rate of surface shear deformation.

When the membrane is forced to maintain a deformed geometry for a long time, the stresses in the membrane relax, and the surface begins to assume a new "equilibrium" geometry.[6] The partial derivative of the log of the material extension with time is given by:

$$\frac{\partial \ln \lambda}{\partial t} = \frac{1}{2}\frac{\partial \tilde{\mathrm{T}}_s}{\partial t}[\tilde{\mathrm{T}}_s^2 + 1]^{-1/2} + \frac{\tilde{\mathrm{T}}_s}{2\tau_c} \qquad (3)$$

where the dimensionless shear resultant $\tilde{\mathrm{T}}_s$ is the ratio T_s/μ, the characteristic time τ_c is the ratio of η_c/μ, and t is time. The creep viscosity coefficient η_c characterizes the rate at which the material stresses relax.

In the flow channel experiment, cells that have adhered to a glass surface are subjected to a fluid shear force.[7] If the force is large enough a strand of membrane (tether) is pulled from the cell body. The force at which tethers begin to form is called the critical force. Originally[8] the critical force was thought to be related to the yield shear resultant of the membrane skeleton. However, recent evidence obtained in our laboratory indicates that tethers may not contain membrane skeleton. These observations support a view that tether formation involves stripping bilayer away from the skeleton. Therefore, the present results are reported in terms of the critical force, and not a yield shear resultant. Regardless of the molecular mechanism involved, the critical force provides a measure of membrane stability and resistance to fragmentation.

The rate at which the tether forms under forces exceeding the critical force is characterized by a viscosity coefficient.[8] For historical reasons we will refer to this as the plastic viscosity of the membrane, even though tether formation may not actually involve plastic deformation of the membrane.

MATERIALS AND METHODS

Intact cells

Cells were obtained from healthy donors either on the morning of an experiment or on the evening before and kept in acid citrate dextrose plasma prior to preparation for the experiment. Cells were separated from the plasma by centrifugation, the buffy coat was discarded, and cells were washed twice in isotonic phosphate buffered saline (PBS) (130 mmol/L NaCl, 25 mmol/L Na_2HPO_4, 6.2 mmol/L KH_2PO_4, 10 µmol/L sodium azide, pH 7.4). The osmolarity of the solutions was measured by freezing point depression and fell within range, 290 to 305 mOsm.

Elevation of the 2,3-DPG concentration was accomplished by the method of Duhm.[9] Packed cells (0.25 mL) were brought to a total volume of 5.0 mL in an incubating buffer containing 10.0 mmol/L inosine, 10.0 mmol/L pyruvate, 50 mmol/L Na_2HPO_4, and 79.5 mmol/L NaCl (300 mOsm, pH 7.4), and kept at 37 °C for two hours. To compensate for the decrease in internal pH due to the elevated concentration of impermeant anion after incubation, the cells were washed twice in phosphate buffered saline at high pH (pH 7.9 to 8.5) then suspended at high pH for measurement. Control cells underwent identical manipulations but were kept in PBS (pH 7.4) for the entire procedure. Cells not used for mechanical measurements were frozen at −70 °C until 2,3-DPG determinations could be made.

Sucrose incubation to deplete cations

In some control measurements, cells were preincubated in low ionic strength sucrose solutions to deplete cells of cations[10] and increase the MCHC under physiologic conditions. Cells were washed twice in PBS then suspended at 5% hematocrit at 37 °C for 40 minutes in a solution containing 5 mmol/L NaCl, 3.0 mmol/L HEPES (pH 7.4), and 240 mmol/L sucrose. The osmolarity of the solution was 300 mOsm. After 40 minutes cells were washed in PBS and suspended in PBS for measurements.

The concentration of 2,3-DPG within the cells was determined enzymatically (see Technical Bulletin No. 665. Sigma Chemical Co, St. Louis, Mo). All reagents were obtained from Sigma. Phosphoglycerate mutase was used to hydrolyse 2,3-DPG to 3-phosphoglycerate, and the amount of released phosphate was determined colorimetrically by the method of Fiske and Subbarow.[11]

The mean cellular hemoglobin concentration (MCHC) was determined by standard techniques. A suspension of red cells was sampled for hematocrit measurement using a capillary tube. An aliquot of the same suspension was taken for hemoglobin determination using Drabkin's solution and absorbance measurements at 540 nm.

Internal pH was determined by measuring the ratio of internal to external chloride concentrations. The method was identical to the one described by Knauf et al[12] with the exception that the cells were equilibrated at 37 °C rather than at 0 °C. ^{36}Cl was used to determine the amount of chloride inside and outside the cells. Cell water was determined by wet and dry weights and corrections for trapped water were obtained by using tritiated inulin as extracellular marker. Given the internal and external chloride concentrations, the hydrogen ion concentration ratio (hence the difference between the internal and external pH) can be calculated from the Donnan equilibrium condition:

$$\frac{[\mathrm{H}^+]_{out}}{[\mathrm{H}^+]_{in}} = \frac{[\mathrm{Cl}^-]_{in}}{[\mathrm{Cl}^-]_{out}} \qquad (4)$$

or

$$\mathrm{pH}_{in} - \mathrm{pH}_{out} = \log_{10}([\mathrm{Cl}^-]_{in}/[\mathrm{Cl}^-]_{out})$$

Ghost preparation

Resealed ghosts were prepared by a method similar to that of Schwoch and Passow.[13] Cells were drawn and washed on the afternoon before an experiment and refrigerated overnight in isotonic phosphate buffered saline. Cells were suspended at 50% hematocrit in 165 mmol/L NaCl and cooled to 0 °C. Thirty-five milliliters (35 mL) of lysing solution (3.5 mmol/L acetic acid, 4.0 mmol/L $MgSO_4$, 0.1 mmol/L phenyl-methyl-sulfonyl-flouride, and 1.0 mmol/L EGTA) were added to 3.5 mL of cell suspension. Care was taken to assure that the temperature remained at 0 °C (± 0.2 °C). After five minutes, 3.5 mL of resealing solution (2.0 mol/L KCl, 25 mmol/L TRIS) was added to the lysate. After five minutes at 0 °C the suspension was transferred to a 37 °C bath and incubated for 45 minutes. After incubation, the ghosts were washed in phosphate buffered saline (300 mOsm, pH 7.4, unless otherwise noted) and suspended in the same buffer for measurements. All mechanical measurements were performed at 37 ± 1 °C.

Measurement of material properties

The surface elastic shear modulus was measured by micropipette aspiration after the method of Evans and La Celle.[14] Cells were aspirated in the dimple region into a micropipette (0.9 to 1.2 μm, ID). The length of the projection in the pipette, L_p, was recorded as a function of the aspiration pressure, ΔP. Evans[15] showed that the relationship between L_p and ΔP is approximately linear when $L_p > R_p$. Chien et al[16] have used the following linear approximation to Evans' original formulation:

$$\frac{\Delta P\, R_p}{\mu} = C_1 \frac{L_p}{R_p} + C_2 \qquad (5)$$

where $C_1 = 2.45$, $C_2 = -0.63$, R_p is the pipette radius, and μ is the surface elastic shear modulus. The shear modulus was calculated from the measured data pairs (ΔP, L_p) by linear regression to Equation 5.

The viscosity coefficient for rapid deformation was measured by the whole cell recovery technique developed by Hochmuth et al.[17] Cells were allowed to settle onto a glass surface and adhere. The adhesion depended critically on the concentration of bovine serum albumin in the suspending medium. The concentration was adjusted in the range 1.0 to 4.0 mg/mL to obtain the desired "stickiness" between the cells and the glass. Cells attached at a single point were aspirated by micropipette at their free edge and extended to approximately twice their resting length by withdrawing the pipette. The cells were released suddenly and the time course of their recovery to their initial geometry was observed. The length-to-width ratio of the cells decreases exponentially with time[17] and can be described by the following relationship:

$$\frac{(L/W) - (L/W)_\infty}{(L/W) + (L/W)_\infty} \cdot \frac{(L/W)_m + (L/W)_\infty}{(L/W)_m - (L/W)_\infty} = e^{-t/\tau} \qquad (6)$$

where $(L/W)_m$ is the largest ratio of length to width and $(L/W)_\infty$ represents the length-to-width ratio in the completely relaxed state. The time constant, τ, is the ratio of the membrane viscosity coefficient to the membrane shear modulus: ($\tau = \eta/\mu$). The length and width of the cell were measured as functions of time, and the time constant was determined by nonlinear least squares regression with two free parameters, the time constant τ and the relaxed length-to-width ratio, $(L/W)_\infty$.

The cells were also tested using the flow channel technique.[7,18] A thin (0.015 mm × 1.0 cm × 4.0 cm) channel was formed with a parafin gasket between a slide and coverglass. The glass in the channel was pretreated with an 8.0% plasma solution. Cells were infused into the channel at a 1.5% to 2.0% hematocrit and allowed to settle on the surface of the channel and remain undisturbed for 30 minutes, during which time some of the cells became attached to the glass. A force was exerted on the cells by fluid moving through the channel. The flow was controlled by adjusting the pressure drop across the channel, and the pressure was monitored using a differential pressure transducer. The magnitude of the force was approximated by the product of the fluid shear stress at the wall times the projected area of the cell. To be consistent with previous flow channel studies,[7,18] a value of 50 μm^2 was used for the projected area of the cell. This corresponds to a disc approximately 8.0 μm in diameter. When the force becomes large enough, a strand of membrane (tether) begins to form between the body of the cell and the attachment site. The rate of tether formation was measured as a function of the applied force. Two parameters were determined from the data: the critical force f_c is the force at which tether growth began to occur, and the plastic viscosity coefficient η_p provides a measure of how fast the tether forms when f_c is exceeded.

The long-term membrane relaxation was measured using the method of Markle et al.[6] Cells were suspended at dilute concentration in phosphate buffered saline (pH 7.4, ~300 mOsm) containing a carefully measured concentration of bovine serum albumin. Cells were aspirated into micropipettes in the dimple region. After a shear modulus measurement was performed the cell was held at a fixed pressure for 20 minutes. Exposure of the cell to light was minimized. After 20 minutes the cell was expelled from the pipette. A residual bump remained on the cell surface. The height of the bump was constant for periods of several minutes after expulsion. The height of the bump was measured within 1 minute after expulsion, as soon as the cell could be recaptured and positioned in the field of view. The ratio of the height of the bump in the pipette before release, Z_0, to the height of the residual bump, Z, gives a measure of the material relaxation (creep) coefficient, η_c. The relationship between Z/Z_0 and time for different values of η_c is given by the solution to Equation 3[6] and is not available in closed form.

RESULTS

Intact Cells

Effects of inosine-pyruvate-phosphate (IPP) incubation. The incubation of intact erythrocytes in IPP caused a dramatic increase in the concentration of 2,3-DPG within the cells. Typically, concentrations increased from about 2.0 to 4.0 mmol/L in control cells to postincubation levels of 17.0 to 24.0 mmol/L. The concentration of chloride within the cells decreased during the incubation because of the increasing concentration of impermeant anion. This causes a decrease in intracellular pH (calculable from the Donnan equilibrium) and an increase in the mean cellular hemoglobin concentration (MCHC) due to the loss of water that accompanies the loss of chloride. To avoid artifactual results due to the effects of changes in intracellular pH, cells with elevated concentrations of 2,3-DPG were suspended in media with high external pH (7.9 to 8.5). The relevant data for solution osmolarity, MCHC, and intracellular pH are given in the tables summarizing the results of each mechanical test.

Shear modulus measurements. Three separate experiments are summarized in Table 1. Because of the strong dependence of the calculated value of the shear modulus on pipette radius and the uncertainty in measuring pipette diameters, valid comparisons between different cell populations can only be made within each experiment. Furthermore, because undetectable small particles lodged in the pipette tip during the experiments could change the effective radius of the pipette, measurements on control cells were performed at the beginning and the end of each experiment, and the average values were compared to ensure that the pipette had not been partially occluded during the course of the experiment. Any difference less than 10% in the value of μ measured at the beginning and end of an experiment was considered satisfactory. In fact, for the data shown in Table 1, the difference was less than 3.0%.

Because cells with elevated 2,3-DPG have higher than normal MCHCs, the first experiment was performed at hypotonic salt concentration. A significant difference was observed between the moduli of cells loaded with 2,3-DPG by incubation in inosine-pyruvate-phosphate and cells incubated in saline (controls); ($p(\mu_1 = \mu_2) = 0.0004$, *t* test). Note

Table 1. Shear Modulus of Intact Erythrocytes as a Function of 2,3-DPG Concentration

Expt No	[2,3-DPG] (mmol/L)	Osmolarity (mOsm)	MCHC (g/dL)	pH_{in}	$\langle\mu\rangle$ (dyn/cm)	SD	n	P ($\mu 1 = \mu 2$)
1	3.2 (control)	262	30.6	7.3	0.0063	0.0018	20	0.0004
	21.3	242	34.4	7.2	0.0044	0.0009	20	
2	Control	242	37.1*	—	0.0057	0.0014	22	0.17
	Control	298	34.5	—	0.0055	0.0018	21	
3	Control	292	—	—	0.0069	0.0010	19	0.16
	15.6	292	46.0	7.2	0.0064	0.0009	16	0.04
	Control	292	42.4*	7.1	0.0058	0.0008	20	

*Elevated by preincubation in sucrose.

that this experiment (Table 1, experiment 1) duplicated (as closely as possible) the conditions of the experiment 2 in Table 2.

The second experiment was performed to see if the decrease in shear modulus was due simply to the change in salt concentration. No difference in shear modulus was observed in control cells over the range of salt concentrations used in the experiments.

The third experiment was performed to test for changes in shear modulus with 2,3-DPG concentrations at physiologic ionic strength. The cells with elevated 2,3-DPG levels were compared to two different control populations: one with a normal MCHC and one with an MCHC that had been elevated by preincubation in low ionic strength sucrose solutions to deplete the cells of cations. The shear modulus for the 2,3-DPG loaded cells was not significantly different from either of the control populations.

In a fourth experiment (not listed in the table) the surface shear moduli of 2,3-DPG-loaded cells was found to be 0.0072 dyn/cm at 290 mOsm (15 cells, SD = 0.0014 dyn/cm) and 0.0054 dyn/cm at 220 mOsm (15 cells, SD = 0.0009 dyn/cm). These results are consistent with the results shown in Table 1, and indicate that the combination of elevated 2,3-DPG concentration and low salt concentration causes a reduction in the membrane shear modulus. The modulus for control cells at 290 mOsm was tested prior to the measurements on 2,3-DPG-loaded cells. The measured value was 0.0065 dyn/cm, SD = 0.0007, n = 10. Unfortunately, the pipette used in that experiment was lost before the last batch of control cells could be tested to check for partial occulsion of the pipette. Because of this, the results of this last experiment are inconclusive in themselves. Nonetheless, they are consistent with, and supportive of, the results given in Table 1.

Viscoelastic recovery. Measured values of the time constant for viscoelastic recovery for intact cells are tabulated in Table 2. Two experiments are summarized in the table. In experiment 1, cells with elevated 2,3-DPG concentrations are compared to control cells at physiologic salt concentration. The time constant for recovery is longer at elevated 2,3-DPG levels. Note that in experiment 1, the increase in external pH overcompensated for the decrease in internal pH brought about by the elevated 2,3-DPG concentration. To see if this overcompensation might have affected the recovery time, measurements were performed on control cells suspended in a buffer with pH 8.5. For the controls at pH 8.5, τ = 0.081s (SD = 0.030s, n = 15). This is not significantly different from the controls measured at pH 7.4 (P = 0.14). This indicates that the high pH was not the major factor causing the increased recovery time.

Table 2. Viscoelastic Recovery Time Constant for Intact Erythrocytes With Elevated 2,3-DPG Concentrations

Expt No	[2,3-DPG] (mmol/L)	Osmolarity (mOsm)	MCHC (g/dL)	pH_{in}	$\langle\tau\rangle$ (sec)	SD	n	P
1	19.7	301	41.0	7.8	0.107	0.054	51	0.000
	4.7	301	36.4	7.2	0.067	0.029	38	
2	19.1	242	36.9	7.2	0.065	0.036	17	0.98
	1.4	242	32.1	7.3	0.065	0.034	17	

During the course of our studies several papers appeared indicating that the recovery time constant for an erythrocyte was increased at high MCHC.[19,20] To check for the effects of increased MCHC in our measurements, experiment 2 was performed at reduced salt concentration to swell the cells and reduce the MCHC. Under these conditions no difference in the recovery time constant was observed between cells with elevated and normal 2,3-DPG concentrations.

Calculation of membrane viscosity. The membrane viscosity coefficient for rapid deformation is equal to the product of the recovery time constant and the membrane shear modulus. The coefficients calculated from the data in Tables 1 and 2 are given in Table 3. At physiologic salt concentrations there is an increase in the apparent membrane viscosity which is probably due to an increase in MCHC. At low salt concentration there is a reduction in membrane viscosity in proportion to the reduction in the membrane shear modulus. The data obtained from erythrocyte ghosts (see Tables 6 and 7) are also included for comparison.

Other viscosity coefficients. Because in our early experiments the viscosity coefficient for rapid deformation at physiologic ionic strength changed in the opposite direction

Table 3. Viscosity Coefficients for Rapid Deformation

[2,3-DPG] (mmol/L)	Osmolarity (mOsm)	μ (dyn/cm)	τ (s)	η (dyn s/cm)
1-5	260-300	0.0063	0.066	4.2×10^{-4}
19.1-21.3	292-301	0.0064	0.106	6.8×10^{-4}
15.6-19.7	242	0.0044	0.065	2.9×10^{-4}
	Ghosts:			
0.0	291-303	0.0053	0.049	2.7×10^{-4}
10	291-303	0.0053	0.050	2.7×10^{-4}

Table 4. Critical Force and Plastic Viscosity Coefficient (Intact Cells)

[2,3-DPG] (mmol/L)				f_c (10^{-6} dyn)				η_p (dyn s/cm)			
Avg	SD	n	pH_{in}	Avg	SD	n	P	Avg	SD	n	P
2.8	1.5	4	7.2	1.00	0.18	17		0.0023	0.0014	16	
22.0	2.3	4	7.4–7.6	0.98	0.19	31	0.80	0.0025	0.0017	31	0.58

to what was expected, additional measurements of membrane properties were made. In the flow channel, strands of membrane (tethers) were formed from cells. No changes in either the critical force or the plastic viscosity coefficient were observed as a result of increasing intracellular 2,3-DPG levels (Table 4).

It should be noted that the results of the flow channel experiment can be affected by the extrinsic geometry of the cells. It is possible that real differences in the intrinsic membrane properties might have been masked by compensating differences in cell geometry between the two cell populations. Cells with high levels of 2,3-DPG tended to be more cupped than control cells, and their projected diameters when at rest in the channel were about 10% smaller than controls. (From the flow channel recordings, we estimated an average diameter of 8.5 μm for controls and 7.8 μm for 2,3-DPG loaded cells. These measurements are probably overestimates of the cell diameter because resolution was limited and measurements were made between outer edges of the diffraction band around the cells. This edge was chosen for measurement because it gave the best contrast and highest reproducibility.) It is not known how the difference in geometry would affect the experimental results because the cupped shape is expected to increase the fluid drag on the cell, whereas the smaller diameter is expected to decrease the drag on the cell. The fact that the cell geometries were similar (although not identical) and the lack of a detectable difference in the flow channel measurements indicate that 2,3-DPG does not significantly affect the "plastic" behavior of intact cells.

The last material parameter measured as a function of 2,3-DPG concentration in intact cells is the time constant for stress relaxation. Markle et al[6] have measured this quantity for normal cells and found it to be a strong function of the bovine serum albumin (BSA) concentration in the suspending medium. In the present experiments (Table 5), the BSA concentration was carefully controlled and constant, and the difference in the residual bump heights for cells with and without elevated 2,3 DPG concentrations was measured. No significant difference in the residual deformation was observed between the two cell populations.

Ghosts

Schindler et al[1] observed an increase in the lateral mobility of membrane proteins in erythrocyte ghosts at elevated 2,3-DPG concentrations. To test for possible changes in membrane material properties under the conditions of those experiments, we have measured the viscoelastic recovery time constant, the surface shear modulus, and the critical force for tether formation of erythrocyte ghosts, with and without 2,3-DPG added to the cells during lysis. The results of these measurements are summarized in Tables 6 through 8.

Table 5. Residual Deformation (Intact Cells)

[2,3-DPG] (mmol/L)				Z/Z_0			
Avg	SD	n	pH_{in}	Avg	SD	n	P
2.0	0.6	6	7.1–7.3	0.58	0.10	12	0.15
20.2	2.0	9	7.4–7.6	0.52	0.13	15	

Ghost morphology and size. Morphologically, the ghosts were nearly all discocytes although there were usually a few echinocytes and cups. The number of echinocytes and cups varied from preparation to preparation, but these nondiscoidal cells were not used for measurements. We estimated the area of cells in the flow channel by taking the product of the length and width of the cells at zero applied force multiplied by $\pi/4$. It should be recognized that because of limited resolution, these measurements are potentially subject to significant errors in absolute magnitude, but they should provide reliable comparisons for the resting diameters of the different cell populations. The projected area of the 2,3-DPG-loaded ghosts was 60.6 μ^2, SD = 10.1 μm^2, n = 107. The projected area of control ghosts was 63.3 μm^2, SD = 9.4 μm^2, n = 59. These were not significantly different by the student *t* test ($P = 0.08$). Precise measurements of cell thickness were not possible, but no obvious difference in the thickness dimensions of the different cell types was observed.

There was no appreciable change in the viscoelastic recovery time constant under the conditions of our measurements (Table 6). The salt concentration of the 16 mmol/L 2,3-DPG samples was elevated because these ghosts were visibly swollen after formation and the higher salt concentration was needed to return them to their normal biconcave shape. (Phosphate was added to two of the samples at the suggestion of Dr M. Sheetz, who pointed out that the accelerative effects of 2,3-DPG on cytoskeletal dissociation that he has observed[2] only occurred in the presence of phosphate.) These

Table 6. Viscoelastic Recovery Time Constant for Erythrocyte Ghosts at Elevated 2,3-DPG Concentrations

[2,3-DPG] (mmol/L)	[PO_4] (mmol/L)	⟨τ⟩ (sec)	SD	n	P
0.0	—	0.049	0.024	69	—
2.0	—	0.054	0.027	33	0.39
4.0	—	0.042	0.021	22	0.22
8.0	—	0.038	0.019	24	0.03
16.0*	—	0.049	0.019	27	0.95
8.0	1.0–3.0	0.052	0.024	33	0.52
16.0*	1.0–3.0	0.050	0.026	26	0.94
0.0*	—	0.052	0.023	15	0.65

*These measurements were performed at elevated salt concentration (~450 mOsm).

Table 7. Shear Modulus of Ghosts at Elevated 2,3-DPG Concentrations

[2,3-DPG] (mmol/L)	Osmolarity (mOsm)	Avg* ± SD (dyn/cm)	Matched RBC Controls ± SD (dyn/cm)	P
0.0	300	0.0058 ± 0.0005	0.0060 ± 0.0003	0.37
10.0	300	0.0051 ± 0.0007	0.0056 ± 0.0011	0.40
0.0	225	0.0048 ± 0.0005	0.0055 ± 0.0005	0.23
10.0	225	0.0054 ± 0.0004	0.0053 ± 0.0008	0.80

*Each value represents an average of five cells.

Table 8. Flow Channel Results for Erythrocyte Ghosts

[2,3-DPG] (mmol/L)	⟨f_c⟩ (10^{-6} dyn)	SD	n	P	⟨η_p⟩ (dyn s/cm)	SD	n	P
0.0	1.05	0.27	15	0.0005	0.00164	0.0014	15	0.78
10.0	0.70	0.24	25		0.00178	0.0017	25	

results are consistent with the results on intact cells at low ionic strength (Table 2) that 2,3-DPG does not affect the viscoelastic recovery time constant.

Unlike the results on intact cells (Table 1), there was no effect of 2,3-DPG (at a concentration of 10 mmol/L)* on the surface shear modulus of erythrocyte ghosts at either physiologic or reduced ionic strength (Table 7). However, there was a significant reduction in the force required to form tethers (Table 8). This is consistent with the observations of Chasis et al,[3] who observed an increase in the rate of fragmentation of 2,3-DPG loaded ghosts as measured in the ektacytometer. Once the threshold was exceeded, the rate at which the tethers formed was not affected by 2,3-DPG, as indicated by the viscosity coefficients shown in Table 8.

DISCUSSION

The observations of Schindler et al[1] on the lateral diffusion of intramembrane proteins and the data of Sheetz and Casaly[2] on the dissociation of erythrocyte membrane skeletons in vitro have provided strong evidence that a high concentration of 2,3-DPG is disruptive to the erythrocyte membrane skeleton. Because the membrane skeleton is the structural basis for the shear elasticity of the membrane and probably accounts for most of the membrane shear viscosity, changes in membrane material properties at elevated levels of 2,3-DPG were expected. Specifically, if a higher fraction of the skeletal proteins were to exist in a dissociated form, one would expect a decrease in membrane stability and possible reductions in the viscosity coefficients for viscoelastic recovery, tether formation, and creep. Consistent with these expectations, a reduction in shear modulus was observed in intact cells with elevated 2,3-DPG levels at subphysiologic salt concentrations, and a reduction in the critical force for tether formation was observed in resealed ghosts. The reduced surface shear modulus, coupled with observations that the viscoelastic recovery time constant did not change, indicate that the membrane viscosity coefficient for rapid deformation also is reduced at low salt concentration. However, no changes were detected in the surface shear modulus or the recovery time constant of ghosts, and no changes were detected in the critical force or the extent of residual deformation in intact cells. Furthermore, the reduction in shear modulus in intact cells was observed only at subphysiologic salt concentrations (240 mOsm) and did not occur under physiologic conditions.

These results demonstrate that lateral mobility is not simply related to the viscosity of the membrane composite as measured by the whole cell recovery experiments. We reach this conclusion in spite of the fact that the values of the membrane viscosity obtained by the two methods are quite similar. Koppel[21] calculated a value of 4.6 × 10^{-4} dyn s/cm from lateral mobility measurements in mouse erythrocytes. This is very close to the values for normal cells obtained in the present investigation (Table 3). This agreement appears to be coincidental. In erythrocyte ghosts, Schindler et al[1] found that the lateral mobility of integral proteins was increased by a factor of 2.5 when the concentration of 2,3-DPG was increased from near zero to 12.5 mmol/L. In contrast, no change in the membrane viscosity of erythrocyte ghosts was detected in the present study at concentrations of 2,3-DPG up to 16.0 mmol/L. The fractional reduction in membrane viscosity observed in intact cells at low ionic strength and elevated (~20 mmol/L) 2,3-DPG concentrations (Table 3, lines 1 and 3) is also significantly less than the fractional change in lateral mobility that Schindler et al[1] observed.

The reason for the discrepancy may be indicated by the results of the flow-channel experiments on ghosts. Preliminary immunochemical evidence obtained in our laboratory indicates that the tethers formed in the flow channel do not contain appreciable amounts of spectrin. That is, it appears that tethers consist primarily of a membrane bilayer that has been separated from the skeletal matrix. If this is so, the ease with which tethers form should depend on the "tightness" of the association between the bilayer and the membrane skeleton. The decrease in critical force that we have observed in ghosts may be indicative of a weakening of the skeletal-bilayer association and could account for the large increase in lateral mobility that has been observed. Weakening of the bilayer-skeletal interaction could also account for the increased rate of fragmentation of ghosts containing 2,3-DPG (10 mmol/L) that has been observed in the ektacytometer.[3]

The effects of 2,3-DPG on the mechanical properties of intact erythrocytes under physiologic conditions appear to be minimal. The increase in membrane viscosity that we have observed is opposite to the behavior one would predict on the basis of the observations of Schindler et al,[1] who measured an increase in the rate of lateral diffusion of membrane proteins at high concentrations of 2,3-DPG. (Increased rate of diffusion should correspond to decreased viscosity and more rapid viscoelastic recovery.) This result is almost certainly due to the increase in MCHC that occurs with increased 2,3-DPG concentrations and not due to the direct influence of 2,3-

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*This concentration was chosen because the increased fragmentation rate of ghosts in the ektacytometer observed by Chasis et al[3] was near-maximal at 10 mmol/L 2,3-DPG.

DPG. Two other laboratories have shown that high cellular hemoglobin concentration increases the viscoelastic recovery time constant of intact erythrocytes.[19,20] The MCHCs in the present experiment are as high as the highest MCHCs in those studies. It is possible that the high MCHC in our experiments is masking a decrease in membrane viscosity caused by the elevated 2,3-DPG concentrations. Because of this, the change in membrane properties that we have measured at subphysiologic salt concentrations may be a more accurate reflection of the direct effects of 2,3-DPG on the membrane. It is surprising, however, that no effect of 2,3-DPG on the viscoelastic properties of ghost membranes was observed at any salt concentration.

A disturbing feature of the present results is the dissimilarity of the effects of 2,3-DPG on intact cells and ghosts. With the exception of the shorter recovery time constants for ghosts (indicative of lower membrane viscosity), the properties of the control ghosts were indistinguishable from the properties of the intact cells. However, none of the effects of 2,3-DPG on intact cells were observed in ghosts and vice versa. This indicates that the effects of 2,3-DPG depend strongly on the conditions of the experiment, and it emphasizes the danger of extrapolating results obtained in one membrane system to another.

The present observation of a change in the membrane shear modulus in intact cells at "low" salt concentration suggests that there is a cooperative effect of high 2,3-DPG concentration and low ionic strength on the membrane properties. Such a cooperative effect could account, in part, for the dissimilar results obtained in the different membrane systems. The reduction of the shear modulus in intact cells was observed at a 2,3-DPG concentration of about 20 mmol/L and a salt concentration of about 240 mOsm. The concentration of 2,3-DPG in the ghost experiments (10 mmol/L) may have been insufficient to produce similar effects at the experimental salt concentration (225 mOsm). It is important to note that the changes in lateral mobility that were observed occurred at salt concentrations below 140 mOsm,[1] and it is possible that changes in the membrane shear modulus of ghosts might occur under those conditions. On the other hand, the change in lateral mobility could be due to a completely separate effect, as indicated by the reduction in the critical force of 2,3-DPG loaded ghosts at physiologic salt concentrations.

The present results confirm the observations of early investigators that under some conditions 2,3-DPG acts to destabilize erythrocyte membrane. Our observations that the effects do not occur under physiologic conditions indicate that the effects of 2,3-DPG in erythrocytes are not physiologically important. However, the recent discovery of spectrinlike and ankyrinlike molecules in a variety of cell types raises the possibility that dissociative effects of 2,3-DPG could have importance in cytoskeletal regulation in other cells. The fact that the effects of 2,3-DPG are most evident in erythrocytes when the MCHC is low or in the absence of hemoglobin (in ghosts) suggests that the effect may be more important in cell types other than erythrocytes. Although the mechanism by which 2,3-DPG achieves its effect is not known, our observations suggest that it could play a role in regulating the interaction between membrane and cytoskeletal elements.

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The Effect of $\overline{\mathrm{C1}}$ Inhibitor Upon Hageman Factor Autoactivation

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Using components purified from human plasma, we have examined the effects of $\overline{\mathrm{C1}}$ Inhibitor ($\overline{\mathrm{C1}}$ INH), the primary inhibitor of activated Hageman Factor (HFa) and Hageman factor fragment (HFf), on Hageman Factor (HF) autoactivation. When Hageman factor was exposed to a negatively charged surface, provided by either a glass cuvette or dextran sulfate, the addition of $\overline{\mathrm{C1}}$ INH gave a dose-dependent inhibition of the activity observed. The ability of $\overline{\mathrm{C1}}$ INH to decrease the maximal enzymatic activity generated was markedly temperature dependent with inhibition increasing as the temperature was raised from 4 °C to 37 °C. Although the rates of both autoactivation and inhibition were decreased at lower temperatures (4 °C), the latter rate was more sensitive to temperature modulation. When HF (final concentration 1 μmol/L) was incubated with C1 INH (0.54, 1.07, and 2.14 μmol/L) in the absence of an initiating surface, no increases in enzymatic activity were observed for up to 48 hours regardless of the $\overline{\mathrm{C1}}$ INH concentration. However, SDS polyacrylamide gel electrophoresis of the incubation mixture revealed that HF autodigestion had occurred by 48 hours despite the presence of C1 INH. In addition, the appearance of a new band suggested that a complex had been formed between the inhibitor and activated HF. Our findings indicate that $\overline{\mathrm{C1}}$ INH does not prevent HF autoactivation but rather inactivates the products of HF autodigestion.

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When human Hageman factor (HF) is activated it converts prekallikrein to kallikrein[1] and Factor XI to Factor XIa[2] to initiate kinin formation and intrinsic coagulation. The main control mechanism for these reactions is the inhibition of activated Hageman Factor (HFa)[3] and Hageman factor fragments (HFf)[4] by $\overline{\mathrm{C1}}$ Inhibitor ($\overline{\mathrm{C1}}$ INH). The mechanism by which human Hageman factor becomes activated initially appears to involve autoactivation[5-7] as well as cleavage by plasma kallikrein[8]; in prekallikrein deficient plasma,[9] autoactivation may be the primary mechanism[10] although normal reciprocal activation of prekallikrein and Hageman factor[7] is much faster.

Since autoactivation is a reaction in which activated Hageman factor (HFa) digests native Hageman factor[5] to form more active enzyme, one might predict that $\overline{\mathrm{C1}}$ INH would inhibit this process by inactivating HFa. However, it is also possible that $\overline{\mathrm{C1}}$ INH interacts with native Hageman factor to inhibit cleavage and, thus, autoactivation. Such a mechanism has been reported for the ability of $\overline{\mathrm{C1}}$ INH to inhibit autoactivation of native $\overline{\mathrm{C1}}$,[11] even though they do not form a demonstrable complex in plasma. This occurs in addition to the known ability of $\overline{\mathrm{C1}}$ INH to inactivate $\overline{\mathrm{C1r}}$ and $\overline{\mathrm{C1s}}$.[12] We have sought to distinguish between these two possible effects upon Hageman factor autoactivation. Our data demonstrate for the first time that $\overline{\mathrm{C1}}$ INH impedes HF autoactivation when it is assessed functionally. It appears to do so by inhibiting the resultant HFa but no demonstrable interaction with HF to prevent its cleavage was observed.

MATERIALS AND METHODS

Reagents were obtained as follows: dextran sulfate (M_r 500,000) and diisopropylfluorophosphate (DFP; Sigma Chemical Co, St. Louis, Mo); N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-pNA (S2222) (Kabi, obtained from Helena Laboratories, Beaumont, Tex); and phenylalanine-phenylalanine-arginine-CMK (Calbiochem-Behring, San Diego, Calif).

Hageman factor (HF) was purified from human plasma using previously published protocols[5,13] and stored at −60 ° in the presence of 10 mmol/L benzamidine. It had activity equivalent to the presence of 1.3% active enzyme. $\overline{\mathrm{C1}}$ Inhibitor ($\overline{\mathrm{C1}}$ INH) was a gift from Dr Peter Harpel, Cornell Medical School, New York, NY. It was also purified from human plasma following the method of Harpel[14] and both preparations were utilized in each experiment with similar results. To minimize any traces of amidolytic activity against S2222 present in the $\overline{\mathrm{C1}}$ INH preparation, it was incubated with 10 mmol/L DFP for one hour followed by extensive dialysis. Prior to use, proteins were dialyzed into 0.1 mol/L sodium phosphate buffer pH 7.0 containing 0.001% Triton X-100.[15] Protein concentrations were determined by the method of Lowry et al[16] using human IgG as a standard.

Amidolytic activity was determined by introducing 5 μL of enzyme into 400 μL of chromogenic substrate S2222 at a final concentration of 0.5 mmol/L in 0.05 mol/L Tris-HCl, 0.001% Triton X-100, pH 7.8, in plastic cuvettes (Walter Sarstedt Inc, Princeton, NJ). The absorbance change at 405 nm was recorded using either a Beckman DU-8 spectrophotometer or a Gilford Model 250 recording spectrophotometer connected to an Apple IIe computer to facilitate data analysis.

Autoactivation in glass cuvettes. Glass cuvettes were cleaned with nitric acid and exhaustively rinsed with distilled water prior to use (Fig 1). In these experiments it was necessary to inhibit any activated Hageman factor (HFa) present in the HF preparation. HF (4.8 μmol/L) was preincubated with 5 μmol/L Phe-phe-arg-CMK for 20 minutes. The final concentration of HF within the cuvettes was 4.3 μg/mL (54 nmol/L), while that of $\overline{\mathrm{C1}}$ INH was either 221 or 110.5 μg/mL (2.1 or 1.05 μmol/L, respectively). Absorbance at 405 nmol/L was continuously recorded for seven hours.

Autoactivation in polypropylene tubes. HF (final concentration 1 μmol/L) was incubated in polypropylene tubes in the presence or absence of C1 INH (2.14, 1.07, and 0.54 μmol/L for up to 48 hours at 37 °C.) Five μL samples were tested for amidolytic activity at 8, 24, 30, and 48 hours. HF was not preinhibited.

Activation in the presence of dextran sulfate (DS). DS 50 μg/mL in 20 mmol/L sodium phosphate buffer pH 7.5 containing 0.001% Triton X-100 was freshly prepared prior to each experiment. HF (final concentration 1 μmol/L) was incubated with DS (final concentration 5 μg/mL) in the presence or absence of $\overline{\mathrm{C1}}$ INH (2.14, 1.07, and 0.54 μmol/L) in polypropylene tubes for up to 24 hours at each of three temperatures (4 °C, 25 °C, and 37 °C). At selected

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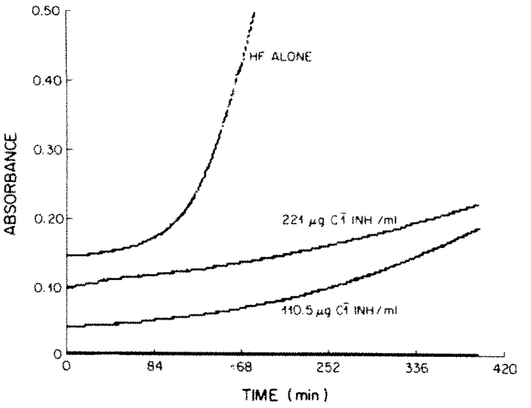

Fig 1. The effect of $\overline{\text{C1}}$ INH on HF autoactivation in glass cuvettes. HF was preincubated with phenylalanine-phenylalanine-arginine-CMK to inactivate HFa and was incubated in the absence or presence of $\overline{\text{C1}}$ INH in glass cuvettes containing S2222 (0.5 mmol/L). The HF final concentration was 4.3 ug/mL (54 nmol/L). The $\overline{\text{C1}}$ INH final concentrations were 221 ug/mL (2.1 umol/L) or 110.5 ug/mL (1.05 umol/L). The absorbance at 405 nmol/L was recorded on a Gilford Model 250 spectrophotometer interfaced with an Apple IIe computer. Initial absorbances are displaced on the Y axis by arbitrary amounts for clarity in presentation.

time points the amidolytic activity of 5 µL samples was determined. HF was not treated with phe-phe-arg-CMK in these experiments.

The data presented as the mean ± 1 SEM. The effects of temperature and $\overline{\text{C1}}$ INH concentration were analyzed by two-way analysis of variance.

SDS polyacrylamide gel analysis of HF cleavage. HF (final concentration 1 µmol/L) was incubated in the absence or presence of $\overline{\text{C1}}$ INH (final concentration 2.2 µmol/L) in polypropylene tubes for 48 hours at 37 °C. In some experiments $\overline{\text{C1}}$ INH (2.2 µmol/L) was added to HF after 48 hours and the mixture was incubated at 37 °C for an additional 30 minutes. As a control $\overline{\text{C1}}$ INH was always incubated alone for 48 hours. At the end of the incubation period, HF amidolytic activity was tested using S2222. Additional aliquots were heated in 0.125 mol/L Tris-HCl (pH 6.8), 12.5% SDS, and 25% glycerol for two minutes at 100 °C under nonreducing conditions and subsequently electrophoresed on polyacrylamide slab gels using the method of Laemmli.[17] The acrylamide concentration was 4% in the stacking gel and 10% in the separating gel. Following overnight fixation, gels were stained with Coomassie brilliant blue (R250).

RESULTS

Previous studies have demonstrated that HF autoactivates in glass tubes requiring incubation for up to two hours,[5] but in polypropylene tubes no such activity is seen during the same time period. However, when HF (1 µmol/L) was incubated at 37 °C in polypropylene tubes for up to 48 hours, a very slow autoactivation was seen, as shown in Fig 1. Little change in enzymatic activity was observed during the first eight hours of incubation and at 48 hours the amidolytic activity measured was only 4-fold greater than that present initially. Under these experimental conditions addition of various concentrations of $\overline{\text{C1}}$ INH (0.54, 1.05, and 2.10 µmol/L) demonstrated comparable inhibitory capacities; ie, even the lowest dose completely prevented the appearance of amidolytic activity and also inhibited the trace of activity seen in the starting material at time zero (Fig 2). Since bacterial growth and resultant endotoxin accumulation can lead to Hageman factor activation, particularly under conditions of prolonged incubation, this experiment was repeated in the presence of both 0.02 mol/L EDTA and 0.02% sodium azide. There was no diminution in the rate of Hageman factor activation in the presence of either bacteriostatic agent.

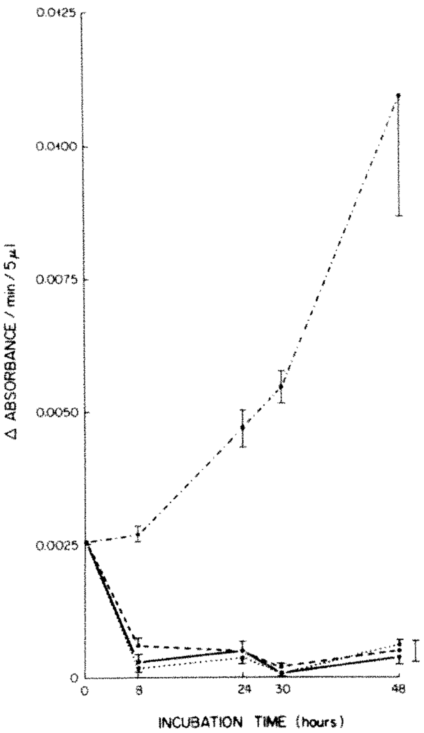

Fig 2. $\overline{\text{C1}}$ INH inhibition of HF autoactivation in polypropylene tubes. HF (1 umol/L) final concentration was incubated in polypropylene tubes with or without $\overline{\text{C1}}$ INH at 37 °C. Amidolytic activity was assayed initially and at 8, 24, 30, and 48 hours. $\overline{\text{C1}}$ INH final concentrations were 2.14 (———), 1.02 (--------), 0.54 umol/L (· · · · · ·), 0 (—.—.—). The data shown are the means ± 1 SEM from 4 separate experiments.

Exposure of native HF to a negatively charged surface by incubation in a glass cuvette accelerates autoactivation.[5] Figure 2 shows a representative experiment using 54 nmol/L HF; in order to observe the process, endogenous HFa was first inactivated by preincubation with 5 µmol/L phe-phe-arg-CMK and then introduced into cuvettes containing substrate (S2222) and different concentrations of $\overline{\text{C1}}$-INH. In the absence of $\overline{\text{C1}}$ INH, autoactivation is evident by two hours shown by the accelerating increase in absorbance. In contrast, when $\overline{\text{C1}}$ INH (1.05 and 2.10 µmol/L) was present, comparable amidolytic activity was not observed within seven hours; the degree of inhibition seen was dependent upon the concentration of C1 INH. For ease of visualization, the three curves are arbitrarily placed along the Y axis; it is the rate of change in absorbance that reflects increasing enzyme concentration. The apparent rate constant of the autoactivation can be quantitated by the slopes of semilogarithmic plots of ΔA/min v time. When examined over the time interval from 90 to 385 minutes, the slope of the secondary plot obtained with 1.05 µmol/L $\overline{\mathrm{Cl}}$ INH was 0.0067 min^{-1}, whereas that obtained with 2.10 µmol/L $\overline{\mathrm{Cl}}$ INH was only 0.0035 min^{-1}.

The temperature dependence of the effect of $\overline{\mathrm{Cl}}$ INH upon surface-induced autoactivation was then examined. In this case, DS provided an alternative source of a negatively charged initiating surface. Incubation of HF (1 µmol/L) with DS (5 µg/mL) in polypropylene tubes resulted in an increase in amidolytic activity which reached a plateau by 60 minutes when examined at ambient (25 °C), physiologic (37 °C), or lowered (4 °C) temperatures.

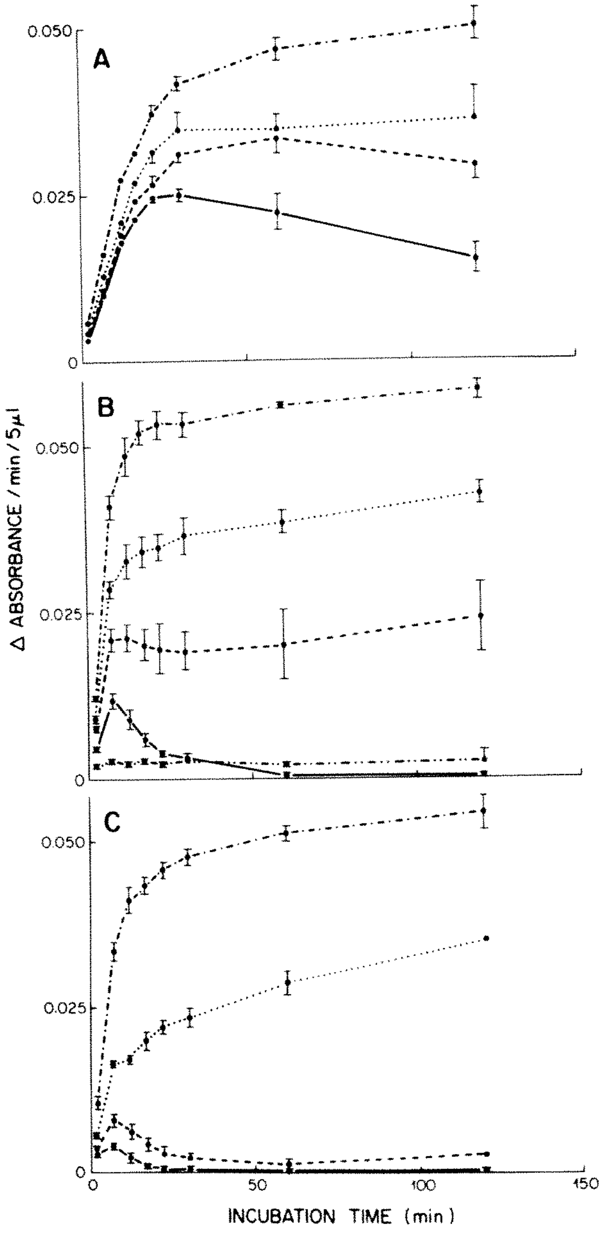

Fig 3. Effects of temperature on the Kinetics of $\overline{\mathrm{Cl}}$ INH inhibition of HF autoactivation with dextran sulfate. HF (final concentration 1 umol/L) was incubated with DS (final concentration 5 ug/mL) in the absence (—.—.—) or presence of various concentrations of $\overline{\mathrm{Cl}}$ INH: 2.1 µmol/L (———), 1.07 µmol/L (--------), or 0.54 umol/L (······). Incubation was for a period of up to two hours at each of three temperatures: (A) 4 °C; (B) 25 °C, and (C) 37 °C. In panel B, an incubation of HF without DS is shown for comparison (—··—··). In these experiments there were between 2 and 6 data points each time.

At 4 °C (Fig 3A) increasing concentrations of $\overline{\mathrm{Cl}}$ INH decreased the peak of enzymatic activity obtained at 30 to 40 minutes. At the highest concentration of $\overline{\mathrm{Cl}}$ INH used, the measured activity declined such that by 24 hours, none remained (data not shown). At 25 °C, (Fig 3B), the same general effect was seen but inhibition by $\overline{\mathrm{Cl}}$ INH was more pronounced and a clear dose response was seen. At the highest concentration, only a transient increase in activity was obtained which then promptly declined. Lesser concentrations of inhibitor appeared to decrease the magnitude of the peak of activity compared to that obtained in the absence of inhibitor. At 37 °C (Fig 3C), the two highest concentrations of $\overline{\mathrm{Cl}}$ INH markedly inhibited autoactivation, while the lowest concentration caused significant inhibition.

Comparison of Figs 3A, B, and C shows that a temperature change from 4 °C to 37 °C had little effect upon autoactivation in the absence of $\overline{\mathrm{Cl}}$ INH. The peak activity reached was similar at each temperature but was delayed somewhat at 4 °C. The data thus indicate that as the temperature is decreased from 37 °C to 4 °C, the rate of inhibition of activated HF by $\overline{\mathrm{Cl}}$ INH decreases, as has been reported previously when activated HF was tested directly.[14] The rate of autoactivation is also lower at 4 °C although it appears slightly greater at 22 °C than at 37 °C. Overall, the decline in the rate of inhibition is greater than that of autoactivation. Thus, the net result of these effects is that as the temperature decreases, the autoactivation reaction partially escapes inhibition by $\overline{\mathrm{Cl}}$ INH and greater transient concentrations of activated enzyme are reached.

In the above experiments we do not attempt to distinguish an effect upon autoactivation from inhibition of activated material in the preparation. The results shown in Figs 1, 2, and 3 could be explained by an interaction of $\overline{\mathrm{Cl}}$ INH with native HF that somehow inhibits digestion. On the other hand, $\overline{\mathrm{Cl}}$ INH might not interact with the substrate for autoactivation, but only with the activated product, (HFa), which is responsible for that digestion. In order to distinguish these possibilities, we examined the products of these reactions by SDS gel electrophoresis under nonreducing conditions. To decrease the rate of reaction, the experiment was performed in polypropylene tubes for a 48-hour time period. The $\overline{\mathrm{Cl}}$ INH was either added at zero time to assess its effect upon autoactivation or it was added after 48 hours of incubation to complex with the activated HF generated by autoactivation. The data are shown in Fig 4. Lane B is the starting HF preparation; lane C contains HF plus $\overline{\mathrm{Cl}}$ INH at time zero; and lane D shows $\overline{\mathrm{Cl}}$ INH alone (mol wt 105,000). In lane E, HF has been incubated alone for 48 hours at 37 °C and complete autodigestion has occurred with significant HFf formation since the gels are run without reduction. In lane F, $\overline{\mathrm{Cl}}$ INH has been incubated with the product of lane E for 30 minutes and complex formation is evident with formation of a doublet at an apparent molecular weight of 131,000 to 133,000. A band just beneath $\overline{\mathrm{Cl}}$ INH is seen that may represent a minor $\overline{\mathrm{Cl}}$ INH form described in plasma[18]

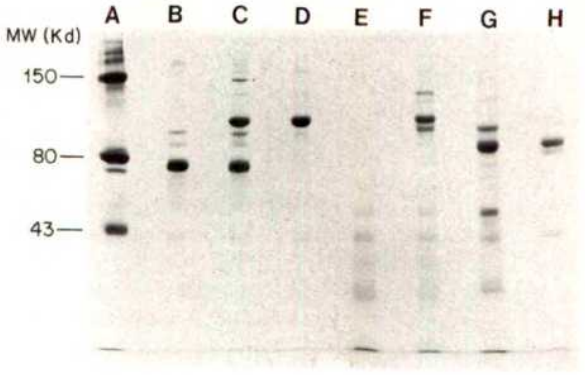

Fig 4. SDS-PAGE of HF autoactivation in polypropylene tubes in the absence or presence of $\overline{\text{C1}}$ INH. HF (final concentration 1 μmol/L), with or without $\overline{\text{C1}}$ INH (final concentration 2.2 μmol/L), was incubated in polypropylene tubes for 48 hours at 37 °C. In some cases, $\overline{\text{C1}}$ INH was added to HF at 48 hours and the mixture was incubated for 20 minutes. In addition, $\overline{\text{C1}}$ INH was incubated by itself for 48 hours. The gel was run under nonreducing conditions. The amount of each protein loaded onto the gel is as follows: MW markers (human IgG, transferrin, and ovalbumin), 2.2 μg each; HF, 2.3 μg; and $\overline{\text{C1}}$ INH, 6.3 μg. Lanes A–H: MW markers (A); HF, time 0 (B); HF and $\overline{\text{C1}}$ INH, time 0 (C); $\overline{\text{C1}}$ INH, time 0 (D); HF, 48 hours (E); HF, 48 hours and $\overline{\text{C1}}$ INH, 30 minutes (F); HF and $\overline{\text{C1}}$ INH, 48 hours (G); and $\overline{\text{C1}}$ INH, 48 hours (H).

and present in our preparation or possibly $\overline{\text{C1}}$ INH cleaved by the HFf.[19] In lane H, containing $\overline{\text{C1}}$ INH alone, two bands are more clearly seen, however after 48 hours incubation a decrease in size of about 16,000 is seen. In lane G, $\overline{\text{C1}}$ INH was incubated with HF for a 48-hour time period. HF autodigestion was not prevented since the band at apparent molecular weight of 78,000 is not seen. The trace residual HF noted compared to lane E or F suggests that autodigestion was slower with $\overline{\text{C1}}$ INH present. A complex is again observed, but it is at 98,000 rather than 133,000, presumably due to the altered mobility of $\overline{\text{C1}}$ INH (lane H). A faint band is also visible at an apparent molecular weight of 122,000.

DISCUSSION

It is clear that $\overline{\text{C1}}$ INH is capable of markedly dampening the autoactivation of Hageman factor that is initiated by interaction with surfaces (Figs 2, 3), as well as the very slow autoactivation that occurs in the fluid phase (Fig 1). The latter reaction may occur in circulating blood, assuming the absence of contact with any "activating" surface. However, when cleavage of Hageman Factor is examined after 48 hours at 37 °C in the absence of a surface, it is clear that cleavage occurs even in the presence of $\overline{\text{C1}}$ INH (Fig 4), ie, the 78,000 molecular weight band is gone. Although the activated Hageman factor that forms is subsequently inactivated (activity is zero at 48 hours), the rate and magnitude of inactivation are not sufficient to prevent digestion of the residual native Hageman factor. Furthermore, the presence of activated forms of Hageman factor suggests that no major interaction with native Hageman factor occurs. Since $\overline{\text{C1}}$ INH prevents C1 cleavage and autoactivation by interaction with the zymogen[11] in addition to its ability to bind the activated enzymes $\overline{\text{C1r}}$ and $\overline{\text{C1s}}$,[12] there is precedent for such a dual interaction. The autoactivation of C1 is intramolecular involving two C1r subunits, and the C1r subcomponent appears to have enzymatic activity in the absence of cleavage. It may be this site with which $\overline{\text{C1}}$ INH interacts. Similar experiments have failed to define an active site in native Hageman factor.[20,21]

If Hageman factor has the potential to be slowly activated in circulating blood, the activated enzyme would likely be bound to $\overline{\text{C1}}$ INH and cleared with synthesis of Hageman factor sufficient to maintain a normal level. Nevertheless, trace quantities of uninhibited enzyme may persist and we have estimated this to be no greater than one molecule in 4,000.[21] This minute level may be sufficient to initiate contact activation when a proper surface is added. Our observations on temperature dependence (Fig 3) in which the effect on autoactivation inhibition is greater than the effect on activation rate may explain in part "cold-dependent" HF activation seen with prolonged plasma incubation at varying temperatures. Further, in a disease such as hereditary angioedema, in which $\overline{\text{C1}}$ INH levels are either low[22] or the molecule dysfunctional,[23] there may be augmented Hageman factor autoactivation. If the active form accumulates, this may predispose to kinin formation[24,25] and attacks of swelling. Studies to test this hypothesis are in progress.

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Ultrastructural Localization of Coagulation Factor V in Human Platelets

By June D. Wencel-Drake, Bjorn Dahlback, James G. White, and Mark H. Ginsberg

The distribution and transport in platelets of human coagulation Factor V was investigated by immunofluorescent and immunoelectron microscopy. In resting intact platelets, little surface staining was observed by immunofluorescence. In permeable resting cells, punctate staining similar to that reported for fibrinogen (Fbg), thrombospondin (TSP), fibronectin (Fn), von Willebrand factor (VWF), B-thromboglobulin (BTG), and platelet Factor 4 (PF4) was observed.[1,2] Double label immunofluorescent staining for Fbg and Factor V demonstrated colocalization, suggesting their presence in the same intracellular structure. Thrombin stimulation induced the appearance of larger (~0.5 μ) immunofluorescent masses of these proteins which exactly colocalized. Thus, at the light level, Factor V and Fbg are localized in the same structure in resting and thrombin-stimulated cells. On the ultrastructural level, an alpha granule localization for Fbg has previously been established.[3-6] We have extended our immunofluorescent observations regarding the localization of Factor V in human platelets by use of transmission electron microscopy of antibody-stained ultrathin frozen sections. In resting cells, staining of virtually all alpha granules was observed for Factor V. In contrast, consistent staining was absent from other organelles including plasma membranes, mitochondria, and vacuolar structures which may represent the open canalicular system. These data thus establish at the ultrastructural level an alpha granule localization of human coagulation Factor V.

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BLOOD coagulation Factor V is a relatively inactive procofactor with Mr = 330,000.[7] Catalytic amounts of thrombin results in limited proteolysis of the single chain Factor V which correlates with a 10- to 80-fold increase in activity.[7] Factor Va functions as an essential nonenzymatic cofactor of the prothrombinase complex by combining with Factor Xa, Ca^{2+}, and the platelet surface (or an alternative phospholipid surface) to catalyze the conversion of prothrombin to thrombin.[8] It has long been recognized that Factor V activity is associated with platelets[9,10] and can be expressed following repeated freeze-thawing of platelets,[9] suggesting an intracellular localization of Factor V. Recently, Chesney et al[11] have demonstrated that release of Factor V induced by collagen is inhibited by antimycin A or 2-deoxyglucose, suggesting that Factor V release is a secretory process. Several lines of evidence have suggested that Factor V is associated with platelet alpha granules: (1) Subcellular fractionation studies indicate that Factor V coisolates with an alpha granule–rich fraction as measured by a functional one-stage assay for Factor V activity[11]; (2) The kinetics of release of Factor V parallels that of platelet factor 4, a known alpha granule protein.[1-6,12-14] In contrast, Gray platelets are selectively deficient in alpha granules,[15,16] yet they have been reported to contain near-normal amounts of Factor V antigen,[17] thereby arguing against an alpha granule localization. The aim of the present study was to establish the subcellular localization of Factor V in the resting cell by use of immunolocalization techniques. In addition, since surface expression of Factor V is required for its procoagulant function, we wished to determine whether the mechanism by which Factor V reaches the cell surface parallels that which has been suggested for alpha granule proteins.[1,2]

MATERIALS AND METHODS

Chemicals. The following were purchased from Sigma Chemicals (St. Louis, Mo): poly-L-lysine, bovine serum albumin, and cacodylic acid. Paraformaldehyde and EM grade glutaraldehyde were obtained from Polysciences (Warrington, Pa). Sepharose 2B and dextran T-10 were obtained from Pharmacia (Piscataway, NJ). Triton X-100 was purchased from J.T. Baker Chemicals (Phillipsburg, NJ). Phosphotungstic acid was purchased from Ted Pella, Inc. (Tustin, Calif).

Platelet preparation. Platelet-rich plasma was prepared from acid citrate dextrose anticoagulated whole blood obtained from healthy aspirin-free volunteers as described.[18] Washed human platelets were subsequently prepared by centrifugation and gel-filtration on Sepharose 2B as described.[19]

Purified proteins. Purified human alpha-thrombin was the generous gift of Dr John Fenton, New York State Department of Health, Albany, NY. This material was diluted to 100 u/mL in modified Tyrode's buffer, stored in aliquots at −70 °C, and thawed once prior to use. Purified human fibrinogen was prepared as previously described.[20] Human coagulation Factor V was prepared according to the method of Dahlback[7] with the following modification: the molarity of the NH_4Cl in the buffer used to dissolve the polyethylene glycol precipitate was lowered to 0.05 mol/L from 0.1 mol/L, to ensure binding to the DEAE-sepharose. The purified material produced a single Coomassie blue staining band of apparent Mr = 330,000, under reducing conditions in 5% to 10% SDS-polyacrylamide gels. Purified Factor V (0.5 mg/mL in 50% glycerol, 25 mmol/L Tris-HCl, pH 7.5, 0.05 mol/L NH_4Cl, 5 mmol/L benzamidine, and 5 mmol/L $CaCl_2$) was labelled with ^{125}I by the lactoperoxidase method of Thorell et al.[21] The labelled Factor V was isolated by gel filtration on a 0.9 × 55 cm column packed with

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Ultrogel AcA 34 in 50 mmol/L Tris-HCl, pH 7.5, 0.1 mol/L NH_4Cl, 0.10 mmol/L benzamidine, 1.0 mmol/L DFP, 10 mmol/L $CaCl_2$ containing 5 mg/mL bovine serum albumin. The labelled Factor V was stored at −20 °C after the addition of an equal volume of glycerol.

Antibodies. Rabbit antihuman Fbg was prepared and characterized as previously described.[2] Rabbit antihuman Factor V, for use in immunofluorescent, immunoelectronmicroscopy, and Western blotting studies, was prepared by immunization of rabbits with our purified single chain Factor V preparation by Dakopatts Inc (Glostrup, Denmark). The antiserum, a concentrated IgG fraction, was immunoadsorbed until a single precipitin line was obtained when tested against plasma in crossed immunoelectrophoresis. Additionally, a single line of identity among plasma Factor V, purified Factor V, and platelet extract was observed in the ouchterlony system. Following adsorption of the antibody with purified Factor V, no immunoprecipitable material was observed when tested against plasma, purified Factor V, or a platelet extract. Rabbit antisera specific for Factor V fragments D and F_1F_2, for use in Western blotting studies, were prepared as previously described.[22]

Western blotting. For Western blotting studies, platelets were prepared as previously described[22] and kept at room temperature in the washing buffer at a cell concentration of 9×10^8 cells/mL. 200 uL aliquots were drawn and pelleted in Eppendorf tubes by centrifugation at 15,600 x g at room temperature for two minutes. The pelleted platelets were dissolved in hot sample preparation buffer containing 3% SDS and 2% B-mercaptoethanol. Identical patterns were obtained when the pellet was first dissolved in a Tris or phosphate-buffer containing 2% Triton X-100, 5 mmol/L diisopropylphosphofluoridate, 1 mmol/L phenylmethylsulfonyl flouride, 10 mmol/L benzamidin, 10 mmol/L EDTA, and 50 ug/mL Leupeptin, and then mixed with the boiling sample preparation buffer.

In previous studies, analysis of purified Factor V revealed a single bank of apparent Mr = 330,000.[7] However, Western blotting analysis of whole platelet extracts revealed several lower molecular weight bands including a doublet at Mr = 220,000 and a band at Mr = 150,000, in addition to the Mr = 330,000 band (Fig 1, lane 1). Subsequent Western blotting analyses of whole platelet extracts using well-characterized anti-Factor V fragment D, or anti-Factor V fragment F_1F_2–specific antibodies[22] demonstrates that the doublet at 200 kd reacts with the F_1F_2 antiserum, indicating that it is the C-terminal half of Factor V, whereas the 150 kd band, reacting with the anti-D antiserum, is the N-terminal half of Factor V (Fig 1, lanes 2 and 3). To test whether Factor V was present in platelets in this form or degraded as a result of platelet lysis, platelet extracts were spiked with ^{125}I-labeled Factor V and run on SDS gels. Western blot analysis of this material revealed bands at Mr = 330,000, 220,000, and 150,000 (Fig 1, lane 4) that exactly corresponded to bands derived from ^{125}I-Factor V on the companion autoradiogram (Fig 1, lane 5). Figure 1, lane 6 confirms that ^{125}I-Factor V existed as the single chain form with an Mr = 330,000, prior to addition to the platelet extract. These data suggest that platelet Factor V may be single chain but becomes proteolysed during the extraction of platelets for electrophoresis, presumably due to platelet-derived proteases, a process that is not fully inhibited by hot sample preparation buffer nor by the inhibitor mixture discussed above.

Immunofluorescence. Immunofluorescent staining of platelets was performed as described.[2] Briefly, resting or thrombin-treated platelets were fixed with 2% paraformaldehyde on ice for one hour. Unreacted aldehyde was blocked with NH_4Cl–Tris buffered saline, pH 7.4, and the cells were permitted to settle on polylysine-coated glass coverslips. In some cases, the cells were treated with 0.1% Triton X-100 for three minutes to render them permeable to antibody prior to staining. The permeable or intact cells were then incubated for 20 minutes with either specific antibody or preimmune rabbit serum. The cells were rinsed with Tris buffered saline containing 0.1% bovine serum albumin, and stained for 20 minutes with fluorescein-labeled goat $F(ab')_2$ antirabbit IgG (Cappel Laboratories, Cochranville, Pa). The cells were counterstained for 20 minutes with a rhodamine conjugated goat $F(ab')_2$ antihuman fibrinogen. The platelets were viewed with a Zeiss Universal microscope equipped with an HBO 50W mercury lamp and IVFI epifluorescence condenser with a BP 546 excitation filter, a KT 580 chromatic splitter, and an LP 590 barrier filter, and were photographed using Tri-X Panchromatic film.

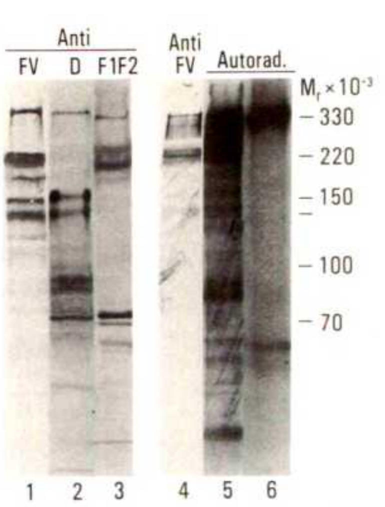

Fig 1. Western blot analysis of rabbit antihuman Factor V antibody. Lanes 1–4 are Western blot analyses, of 5% to 15% SDS-polyacrylamide gels run under reducing conditions, using either rabbit antihuman Factor V (lanes 1 and 4), rabbit antihuman Factor V fragment D specific (lane 2), or rabbit antihuman Factor V fragment F_1F_2–specific (lane 3) antibodies. Peroxidase-labelled goat antirabbit IgG was employed as the secondary antibody. Lanes 1–4 are blots of pelleted washed platelets (~5×10^7/lane) that were dissolved in boiling sample preparation buffer as described in Materials and Methods. Lanes 4 and 5 show the Western blot and companion autoradiograph of a platelet extract that had been spiked with an ^{125}I-labelled tracer of single chain human Factor V prior to boiling with sample preparation buffer. The autoradiography of the tracer mixed with cold human Factor V is seen in lane 6. The relative molecular weights of the components are indicated at the right.

Fixation and cryoultramicrotomy. Fixed platelets for sectioning were prepared by adding a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde at 37 °C to a suspension of washed cells in 0.1 mol/L $NaPO_4$, pH 7.4, at 37 °C. The cells were then mixed and placed on ice for 15 minutes at 4 °C. Unreacted aldehyde was blocked by treatment with NH_4Cl–Tris buffered saline at pH 7.4 and the cells were subsequently embedded in a low gelling temperature agarose. Agarose blocks were infused with 0.8 mol/L sucrose as cryoprotectant for 30 minutes, mounted on copper bullets, and stored in liquid nitrogen until sectioning.

Sectioning was performed at −84 °C according to the method of Tokuyasu and Singer,[23,24] utilizing a Sorvall MT 2-B ultramicrotome with an LTC-2 cryokit attachment (DuPont Instruments, Sorvall Biomedical Division, Newton, Conn). Sections ranging in thickness between 800 and 1000 angstroms were transferred to a parlodion and carbon-coated copper grid by means of a thin wire loop containing a drop of 2.3 mol/L sucrose, which thawed en route to the grid. Half micron frozen thick sections, for immunofluorescence, were obtained by sectioning at −54 °C. Sections were transferred to acid washed glass slides and stained as described for whole cells.

Immunolabelling and negative staining. Rabbit antihuman Factor V or preimmune rabbit serum were used as primary antibodies in an indirect immunolabelling procedure. Affinity purified and biotinylated goat antirabbit IgG (Hyclone Laboratories, Logan Ut) was used as the second antibody. Antibody-antigen interactions were visualized by means of a colloidal gold-avidin conjugate.

Five nanometer colloidal gold-avidin was prepared as previously described.[6] Prior to immunolabelling, the thawed ultrathin frozen sections were conditioned with 2% gelatin and 0.1% bovine serum albumin. Following completion of immunolabelling, the sections were negatively stained with a mixture of 0.2% phosphotungstic acid and 1% dextran. Sections were viewed on a Hitachi 12-A transmission electron microscope with an accelerating voltage of 75 KV, and were photographed with Kodak electron microscope film No. 4489.

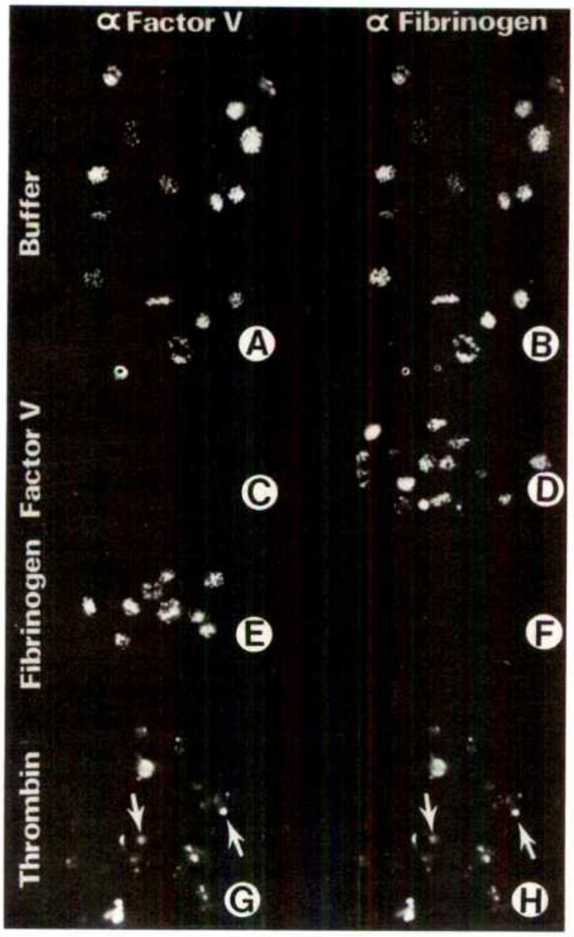

Fig 2. Colocalization of Factor V and Fbg in resting and thrombin-stimulated platelets. Resting, permeable platelets were stained with a mixture of rabbit antihuman Factor V and rhodaminated goat F(ab′)$_2$ antihuman Fbg and counterstained with fluoresceinated goat F(ab′)$_2$ antirabbit IgG. Antibodies absorbed with buffer alone (A and B) demonstrated punctate intracellular staining for Factor V which exactly colocalized with Fbg. Incubation of the antibody mixture prior to staining with either purified Factor V (C and D) or Fbg (E and F) only blocked fluorescence when an antibody was absorbed with its parent antigen. When platelets were fixed one minute following thrombin stimulation, we noted the appearance of large, peripheral immunofluorescent masses which are defined as smooth fluorescent structures ≥ 0.5 μm in diameter and which double stained for Factor V and Fbg (G and H, arrowheads); Original magnification, ×1,400; Current magnification, ×1,050.

RESULTS

Localization of Factor V in resting and thrombin-stimulated platelets. Intact resting platelets demonstrated minimal surface staining. When cells were made permeable with Triton X-100, however, punctate immunofluorescent staining for Factor V and Fbg was observed. Antibody specificity was documented in double-label immunofluorescent cross-blocking studies. In these studies, mixtures of rabbit antihuman Factor V antibody and rhodaminated goat F(ab′)$_2$ antihuman Fbg were absorbed overnight at 4 °C with either a 1.4-fold molar excess of purified Fbg, or an equimolar concentration of purified Factor V or buffer. Permeable platelets were subsequently stained with these absorbed antibodies and counterstained with fluoresceinated goat F(ab′)$_2$ antirabbit IgG. As shown in Fig 2A and B, antibodies absorbed with buffer alone demonstrated punctate intracellular staining for Factor V which exactly colocalizes with Fbg. However, when platelets were stained using the absorbed antibodies, fluorescence was blocked only when an antibody was absorbed with its parent antigen, confirming specificity of staining (Fig 2C and F). Additionally, control testing with preimmune rabbit or goat IgG failed to stain either intact or permeable cells (data not shown).

To determine the localization of Factor V antigen immediately following thrombin stimulation, cells fixed after one minute's stimulation at 24 °C were double stained for Factor V and Fbg. Thrombin stimulation resulted in the time-dependent translocation of Fbg and Factor V into larger immunofluorescent masses similar to those that have been reported for TSP, Fn, VWF, BTG, and PF4 (Fig 2G and H).[1,2] These masses were defined as smooth fluorescent structures, ≥ 0.5 microns in diameter, and were not seen in

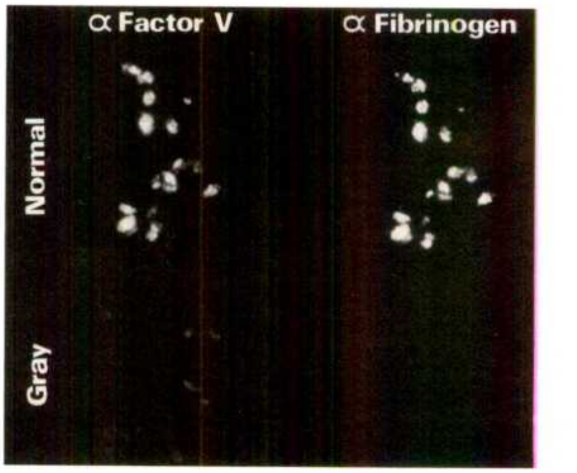

Fig 3. Reduced immunofluorescent staining for Factor V and Fbg in Gray platelets. Frozen thick sections of normal and Gray platelets were double stained for Factor V and Fbg as described in Materials and Methods. The left panels illustrate Factor V staining and the right illustrate Fbg staining of the same field of platelets. Normal cells are depicted in the upper panels and gray platelets are shown below. Original magnification, ×1,400; Current magnification, ×770.

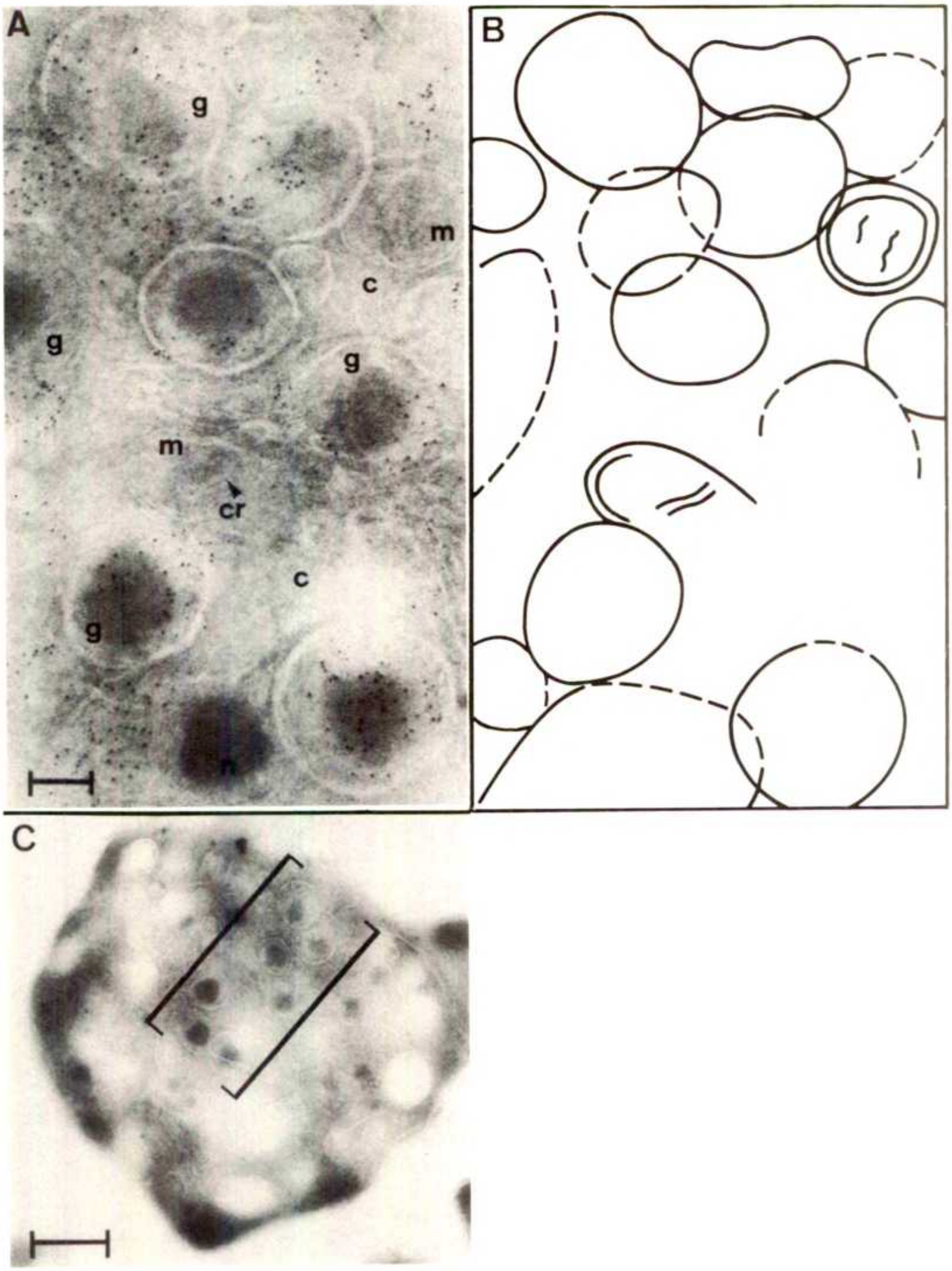

Fig 4. Indirect immunolabeling of Factor V in cryostat sections of human platelets. Ultrathin frozen sections were incubated with rabbit antihuman Factor V as the primary antibody. Biotinylated goat antirabbit IgG was employed as the second antibody. Antibody-antigen interactions were visualized by means of a 5 nm colloidal gold-avidin conjugate. Panel A, a magnification of the whole cell shown in panel C, demonstrates heavy labeling over structures identified as alpha granules (g) with minimal labeling of mitochondria (m), cristae (cr), or the platelet cytosolic matrix (c). Panel B represents a corresponding illustration, with intact membranes designated by solid lines and presumptive membranes, by dashed lines, (original magnification (A), 107,500 X, bar 0.1 μm; current magnification, 80,625 X; (B) original magnification, 107,500 X; current magnification, 80,625 X; (C) original magnificationm 28,000 X, bar 0.5 μm; current magnification, 21,000 X).

resting cells. The above data suggested that Factor V and Fbg are stored in the same intracellular organelle and are processed to the cell surface via common structural intermediates.

As an alpha granule localization for Fbg has been established,[3-6] it seemed likely that platelet Factor V would also be stored in alpha granules. To examine this possibility frozen thick sections of gray platelets, which are selectively deficient in alpha granules,[15,16] were double stained for Factor V and Fbg. As seen in Fig 3, immunofluorescent labeling of gray platelets revealed markedly decreased staining for both Factor V and Fbg (lower panels) when compared to normal platelets (upper panels).

Ultrastructural localization of Factor V in frozen thin sections. To investigate the ultrastructural localization of Factor V by transmission electron microscopy, we prepared negatively stained, ultrathin frozen sections of resting platelets. Platelet morphology in negatively stained cryosections has been described previously.[6] Alpha granules are identified on the basis of their size, 0.1 to 0.4 u, roughly spherical shape, single limiting electron lucent membrane of 100 ± 21 angstroms in thickness, and the occasional appearance of a darkly staining eccentric nucleoid. Other identifiable subcellular organelles include mitochondria with characteristic double membranes and cristae.

Indirect immunolabelling of frozen sections with an anti-Factor V antibody demonstrated heavy labelling of structures identified by the above criteria as alpha granules (Fig 4A). The labelling pattern was neither restricted to the nucleoid nor to the electron lucent periphery, but rather appeared inhomogenous. In contrast, label was absent from plasma membrane and mitochondria. Only background levels of staining were associated with the platelet cytoplasmic matrix. In contrast, control sections, stained with preimmune rabbit serum, demonstrated only occasional random colloidal gold particles (not shown).

DISCUSSION

In the present study we have investigated the intracellular localization of human coagulation Factor V and its initial redistribution in response to thrombin. Utilizing immunofluorescence techniques we have found that Factor V and Fbg colocalize in the interior of resting platelets. On the ultrastructural level, Factor V was localized within structures morphologically identified as alpha granules by means of indirect immunolabeling of ultrathin frozen sections. Following thrombin stimulation, Factor V is consolidated into larger structural intermediates which double stain for Fbg.

It has long been recognized that Factor V is an essential cofactor in the assembly of the prothrombinase complex.[8,25] Activated Factor V (Factor Va) functions as the platelet-bound receptor for activated Factor X (Factor Xa).[26] While the platelet receptor for Va does not require platelet release for its expression,[26] platelet Factor V contributes to functional prothrombinase complex assembly.[17] In this regard, subcellular fractionation studies have suggested an alpha granule localization for Factor V.[11] However, in recent studies Tracy et al[17] have suggested that normal Factor V levels are present in Gray platelets (which are selectively deficient in alpha granules).[15,16] While these results may represent partially activated platelets with surface-bound Factor V or reflect heterogeneity among gray platelet patients (as the patient we examined demonstrated markedly reduced Factor V antigen as judged by immunofluorescence), the present studies and the subcellular fractionation studies of Chesney et al[11] localize the bulk of Factor V antigen in the resting cell to alpha granules. Additionally, following thrombin stimulation, Factor V was shown to become consolidated into larger structural intermediates that double stain for Fbg and have been suggested to represent compound granules.[1] Thus, intraplatelet Factor V is apparently stored and processed to the cell surface by a mechanism common to a growing number of alpha granule proteins.[1,2] Whether the apparent functional differences between platelet and plasma Factor V[17] are due to structural differences, as has been shown for von Willebrand factor, another alpha granule protein,[27] or simply reflect a local concentration effect brought on by a fusing compound granule, remains to be evaluated.

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An In Vitro Model of Erythroid Egress in Bone Marrow

By Richard E. Waugh and Mohamed Sassi

An in vitro system has been developed that mimics the passage of erythrocytes from the bone marrow to the circulation. Bone marrow egress and its proper regulation are vital physiologic processes. However, because of the inaccessibility of the marrow, it is difficult to evaluate the various factors important in controlling these processes or even to define the precise mechanism by which egress occurs. The in vitro system has been designed to evaluate the importance of different physical parameters in regulating egress. It consists of a thin silicon wafer (thickness ≃ 1.0 μm) cemented over the tip of a large (15.0 μm ID) micropipette. The wafer contains a single circular pore. Cells were observed under the microscope as they passed through the pore under controlled pressures. The rate and duration of passage were obtained from videorecordings of the experiment. The measured passage times agreed well with the predictions of a simple analytical model of a cell passing through a thin aperture. The experimental results confirm the conclusion reached from the analysis that the pressures needed to drive a cell through the pore are well within the physiologic range, and the time needed to complete egress is typically less than 1.0 seconds. These results support the hypothesis that erythrocyte egress may be driven by a hydrostatic pressure difference across the pore.

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AN average adult man has approximately 2.5 × 10^{13} red blood cells in circulation at any given time, and the average lifespan of each cell is approximately 120 days. To maintain homeostasis, the bone marrow must supply about 2.5 × 10^6 new erythrocytes to the circulation every second. To get from the hemopoietic space, where the cells grow and differentiate, to the marrow sinuses, which are contiguous with the circulation, the cells must pass through small apertures in the endothelial barrier separating the two compartments. These apertures are generally less than 1.0 μm in thickness and range in diameter from 1.0 μm to 3.0 μm.[1] The mechanism by which the cells pass through these apertures and the factors that regulate the flux of cells across the endothelial boundary are not well understood. We have developed an in vitro model of the egress process to evaluate the importance of different physical parameters in controlling this process.

The motivation for these investigations came from examining electron micrographs of reticulocytes in passage through endothelial pores in the bone marrow of the mouse (Fig 1). The spherical contour of the portion of the cell in the sinus suggested to us that reticulocyte egress may be driven by a hydrostatic pressure difference across the pore. An approximate analysis was undertaken[2] to obtain estimates for the time it should take a cell to complete egress at different pressures and to evaluate the importance of pore diameter and membrane viscoelastic properties on the rate of egress. Based on this analysis, it was concluded that there is a threshold pressure on the order of 1.0 to 3.0 mmHg below which the cell would not pass through the pore because of the elastic resistance of the membrane to deformation. It was also concluded that for pressures appreciably greater than the threshold pressure, the time required for the cell to complete its passage was short, typically less than 0.5 seconds.

In the present paper an experimental system has been developed to test the analytical predictions. A thin (~1.0 μm) silicon wafer with a single pore in it was cemented with epoxy over the tip of a large (15.0 μm ID) micropipette, and single erythrocytes were observed as they passed through the pore. The time course of the passage was observed as a function of the driving pressure. The experimental results agreed well with the analytic predictions. These observations support the hypothesis that erythrocyte egress may occur through preexisting endothelial pores and be driven by hydrostatic pressure.

MATERIALS AND METHODS

Cells were obtained by finger prick and suspended in a buffered saline solution containing 125 mmol/L NaCl, 24.4 mmol/L Na_2HPO_4, 6.1 mmol/L KH_2PO_4, 1.0 μmol/L sodium azide, and 3.0 mg/mL bovine serum albumin. The cell suspension was placed on the stage of an inverted microscope (Nikon M) in a chamber (1.0 × 1.0 × 0.1 cm) that allowed access for micropipettes from opposite sides. Experiments were performed at room temperature (~25 °C). The pipette-pore was connected via continuous water connection to an enclosed reservoir. Zero pressure across the pore was set by positioning the water-filled reservoir such that particles in or near the opening remained stationary. Changes in pressure relative to zero were produced by pressurizing the enclosed reservoir with an air-filled syringe. The pressure was monitored with a differential pressure transducer. The pipette-pore was introduced into the chamber on the microscope stage and an ordinary micropipette was introduced from the other side. The second pipette was used to manipulate cells from the bottom coverglass to a place near the pore opening. The cell was sucked into the pipette-pore, then a constant outward pressure was set using the air-filled syringe. The passage of the cell out of the pore under constant pressure was observed, then the cell was reaspirated into the pore and the expulsion was repeated at a different pressure. Several passages at different pressures were observed for each cell. Experiments were recorded on videotape along with a digital display of the trans-pore pressure and the time. If the pressure varied during the course of the passage the data for that passage were disregarded. The height of the cell projection outside the pore was obtained from the recordings by frame-by-frame analysis. The total time for passage at the different pressures was also noted.

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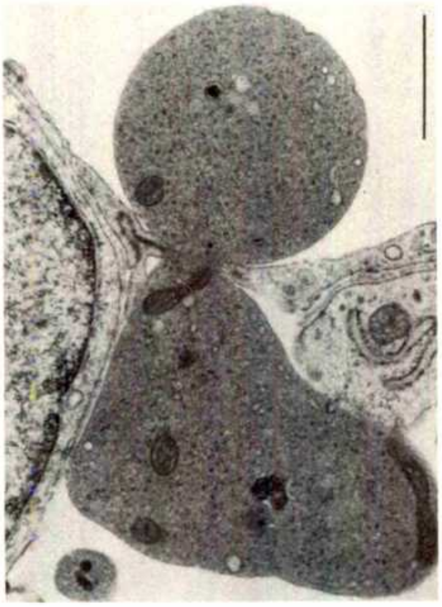

Fig 1. **This transmission electron micrograph shows the spherical projection of a reticulocyte into the blood sinus during egress in the bone marrow of a mouse (Bar = 1.0 μm). (Reprinted with permission.[1])**

The pipette-pore assembly. The assembly process is depicted diagramatically in Figs 2(A–D). The silicon wafer pore was manufactured at the National Resource and Research Facility for Submicron Structures at Cornell University. Wafers were thinned to a thickness of approximately 1.0 μm, and a pattern was etched through the wafer (Fig 2A). Pipettes were formed from microneedles pulled on a David Kopf Instruments pipette puller. The needles were ground to a flat tip against an aluminum oxide surface in an ultrasonic bath. The intensity of the bath was adjusted using a Variac transformer. After a flat tip of the desired diameter was formed, the pipette was bent at a 90° angle using a microforge (Stoelting) mounted on the stage of the microscope (see Fig 2B).

To attach the pore to the pipette, the wafer was placed on a glass slide on the microscope stage, and a ring of ultraviolet-sensitive adhesive (Crystal Clear Adhesive, Loctite Corporation, Cleveland, Ohio) was painted around the pore to match the pipette circumference. The pipette was positioned such that the opening faced toward the objective and was centered over the pore (Fig 2C). The pipette was lowered onto the wafer with a micromanipulator until it contacted the glue and formed a seal. The assembly was exposed to ultraviolet light to harden the glue. Then using the micromanipulator the pipette was pressed down onto the wafer until the pore was punched out of the wafer along the perforated ring (Fig 2D).

Prior to the experiment, the pipette-pore assembly was filled with filtered, phosphate-buffered saline. The pipette-pore was inserted into a sealed, saline-filled chamber and the chamber was pressurized by depressing the plunger of a syringe, forcing saline through the pore into the tip of the micropipette. When the tip was filled, the pipette-pore was removed from the chamber and the shaft of the pipette was filled from the back using a 31 gauge needle.

Theoretical background. An approximate analysis of an erythrocyte passing through a thin, circular aperture has appeared elsewhere,[2] and only a brief description of the analysis will be given here. The geometry used to approximate the cell contour is shown in Fig 3. The undeformed membrane consists of two flat disks of radius R_{Do}, connected at the edge. To simulate egress, the upper disk is deformed into a spherical section. The area of the surface is conserved, but the cell volume is allowed to change freely. (Volume conserving models proved to be analytically intractable.) As the

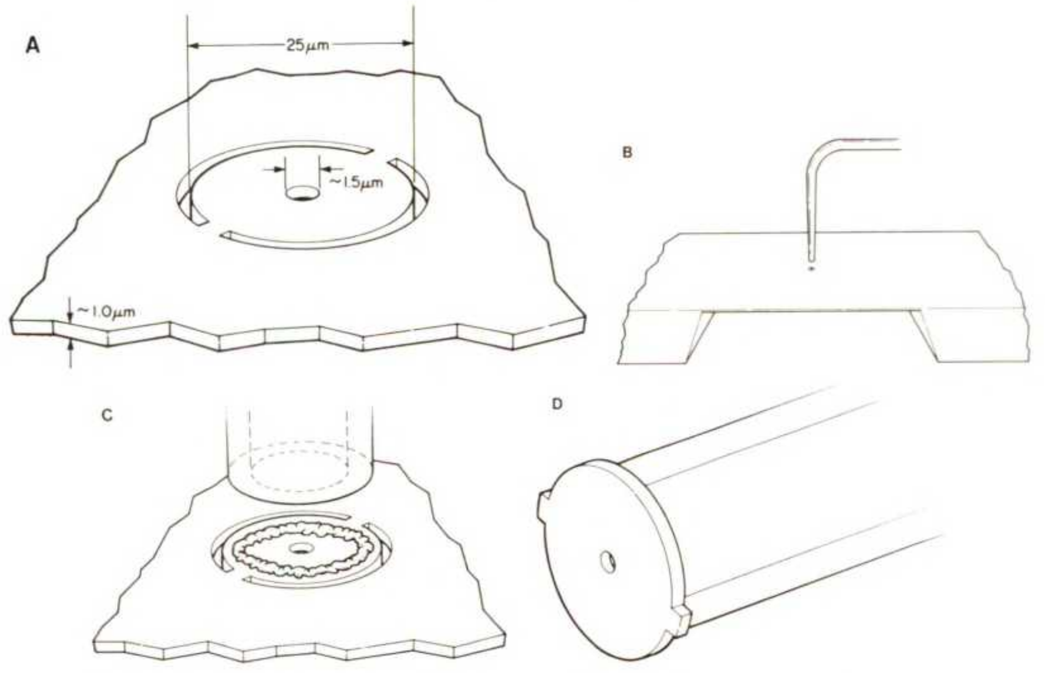

Fig 2. **This series of schematic drawings shows the procedure for assembling a pipette-pore. (A) A pattern is etched through a thin silicon wafer. The pore is at the center of a solid disk with two tabs holding the disk to the rest of the wafer. (B) A glass micropipette is bent to a 90° angle and the mouth of the pipette is centered above the pore. (C) A second pipette is used to paint a ring of adhesive onto the silicon disk where the face of the pipette will come to rest. (D) After the adhesive has cured, the disk is pressed out of the wafer and removed. The final assembly is shown.**

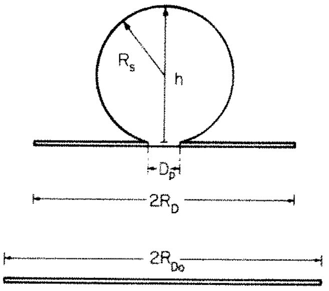

Fig 3. A schematic drawing of the geometric model used in the analysis. The initial cell radius is R_{Do}. The pore diameter is D_p. The radius of the spherical projection is R_s and its height is h. The instantaneous cell diameter, R_D, decreases with time as more material passes through the pore. (Reprinted with permission.[2])

spherical projection grows, material is drawn from the lower disk and radially across the upper disk to the pore edge (radius = R_p). When the radius of the lower disk equals R_p, egress is complete.

A simple viscoelastic constitutive equation is used to describe the membrane material behavior[3]:

$$T_s = \frac{\mu}{2}(\lambda^2 - \lambda^{-2}) + 2\eta V_s \tag{1}$$

where T_s is the maximum shear resultant at a material point, μ is the surface shear modulus, λ is the material extension in the radial direction relative to the unstressed geometry, η is the membrane surface viscosity, and V_s is the rate of material deformation. Values for μ and η were taken from the literature: $\mu = 0.007$ dyn/cm[4] and $\eta = 0.0005$ dyn s/cm.[5] The resistance of the cell to entering the pore is due to the viscoelastic deformation of the planar disk surface as it is drawn toward the aperture. In the planar region, the balance of forces in the radial direction is given by,[6]

$$\frac{dT_m}{dr} = \frac{-2T_s}{r} \tag{2}$$

where T_m is the principal membrane tension in the radial (meridional) direction and r is the radial coordinate. A differential equation is obtained by combining Equations 1 and 2 to eliminate T_s. Solution of the equation requires two boundary conditions. At the edge of the pore ($r = R_p$) we take $T_m = \Delta PR_s/2$, where R_s is the radius of the spherical section that has passed through the pore and ΔP is the pressure difference across the pore. At the edge of the cell ($r = R_D$) we take $T_m = 0$. The solution to the integration results in a first order differential equation relating the height of the spherical projection, h, to time. In terms of dimensionless quantities this takes the form:

$$\frac{d\bar{h}}{d\bar{t}} = \left\{\Delta\bar{P}\left(\bar{h} + \frac{1}{\bar{h}}\right) - \frac{\bar{h}^2}{2} - \ln\left[\left(1 - \frac{\bar{h}^2}{\bar{R}_{Do}^2}\right)(\bar{h}^2 + 1)^{1/2}\right]\right\} \cdot \left[2\bar{h} - \frac{4\bar{h}}{2\bar{R}_{Do}^2 - \bar{h}^2}\right]^{-1}, \quad A_s \le A_{1/2} \tag{3}$$

and:

$$\frac{d\bar{h}}{d\bar{t}} = \left\{\Delta\bar{P}\left(\bar{h} + \frac{1}{\bar{h}}\right) + 1 - \bar{R}_{Do}^2 - \frac{\bar{h}^2}{2} + \frac{1}{2}\ln\left[2\bar{R}_{Do}^2 - \bar{h}^2 - 1\right]\right\} \cdot \left[2\bar{h} - \frac{4\bar{h}}{2\bar{R}_{Do}^2 - \bar{h}^2}\right]^{-1} \quad A_s > A_{1/2} \tag{4}$$

where A_s is the area of the spherical section and $A_{1/2}$ is half of the total cell area.

The variables in these expressions are

$$\bar{h} = h/R_p$$
$$\Delta\bar{P} = \Delta PR_p/4\mu$$
$$\bar{R}_{Do} = R_{Do}/R_p$$
$$\bar{t} = t \cdot \mu/\eta$$

where ΔP is the pressure across the pore, R_p is the pore radius, R_{Do} is the radius of the undeformed disks, and h is the height of the spherical section (see Fig 3). Two expressions are required because of the different relationships between λ and r for material that was originally on the upper or lower disk. Before half of the cell passes the pore edge, the integral encompasses regions from both the upper and lower disks (Equation 3). After half of the cell passes the pore edge (Equation 4), only material originally from the lower disk remains in the planar region.

Equations 3 and 4 were integrated numerically using Simpson's Rule to obtain the height of the spherical projection as a function of time, as well as the total time for egress for a given set of parameters, ΔP, R_{Do}, and R_p. It is important to recognize that there is a threshold pressure, ΔP_{min}, below which egress does not occur.[2] Mathematically this occurs because for $\Delta P < \Delta P_{min}$, dh/dt becomes zero before egress is completed; hence the integration of Equations 3 and 4 cannot be completed. Physically this occurs because the pressure must be sufficient to overcome the elastic resistance of the mem-

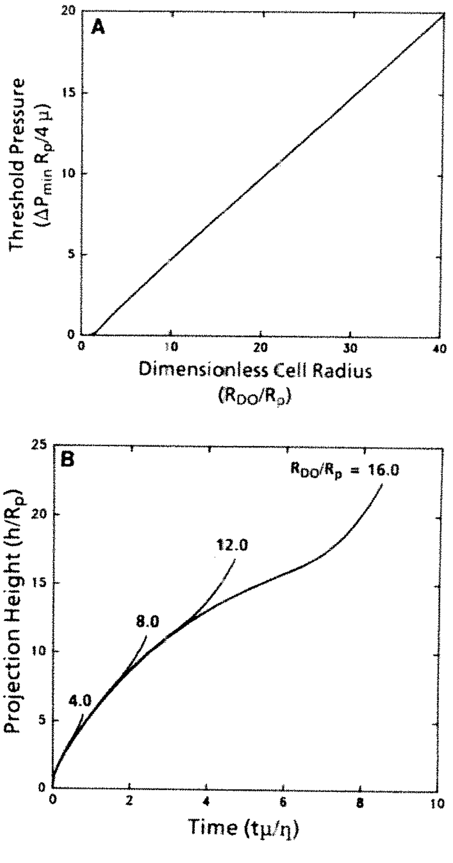

Fig 4. Results of the Analysis. (A) The dependence of the dimensionless threshold pressure ($\Delta PR_p/4\mu$) on the dimensionless cell radius (R_{Do}/R_p). Note that for a cell radius of 4.0 μm and a pore radius of 0.5 μm ($\bar{R}_{Do}$ = 8.0) the minimum pressure for egress is less than 2.0 mm Hg (2.7 × 10^3 dyn/cm^2) ($\Delta\bar{P}$ ~ 4.75). (B) The effect of changing $\bar{R}_{Do}$ on the time course of egress. $\Delta\bar{P}$ is held constant ($\Delta\bar{P}$ = 10.0). For larger values of $\bar{R}_{Do}$ the final value of $\bar{h}$ is larger because the membrane has more surface area.

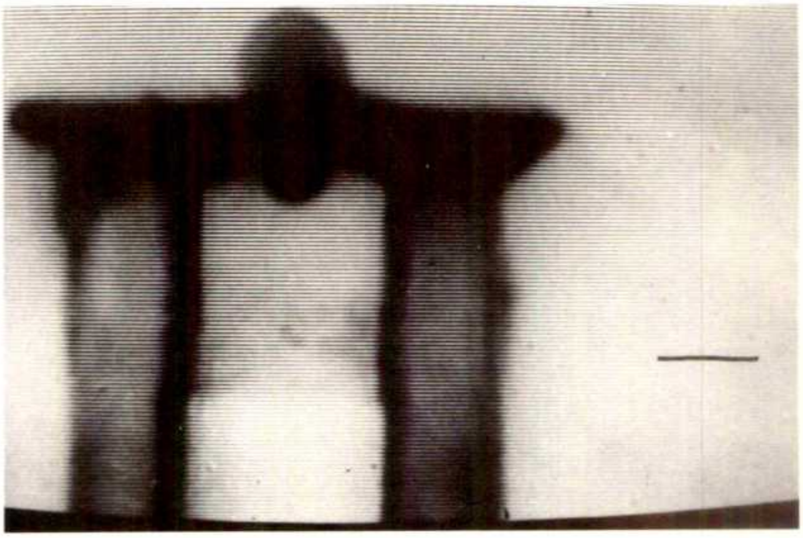

Fig 5. A photograph taken from the television monitor showing a cell passing through the pipette-pore. The driving pressure was 1.87×10^3 dyn/cm², and the total time to complete the passage was approximately 0.3 seconds (Bar = 5.0 μm).

brane. The dependence of ΔP_{min} on the dimensionless cell radius (R_{Do}/R_p) is shown in Fig 4A. When ΔP_{min} is exceeded, the cell passes through the pore. The time course of the passage also depends on the dimensionless cell radius, as is shown in Fig 4B.

Several assumptions are implicit in the analysis. The viscous resistance of the cytoplasm and the bending stiffness of the membrane are neglected. The interaction between the membrane and the pore is assumed to be frictionless. The surface geometry is constrained, no folding or wrinkling of the surface is allowed. Finally, the volume of the cell is allowed to change freely, and the restrictions to passage that a real cell might experience because of its constant surface area and volume are not considered.

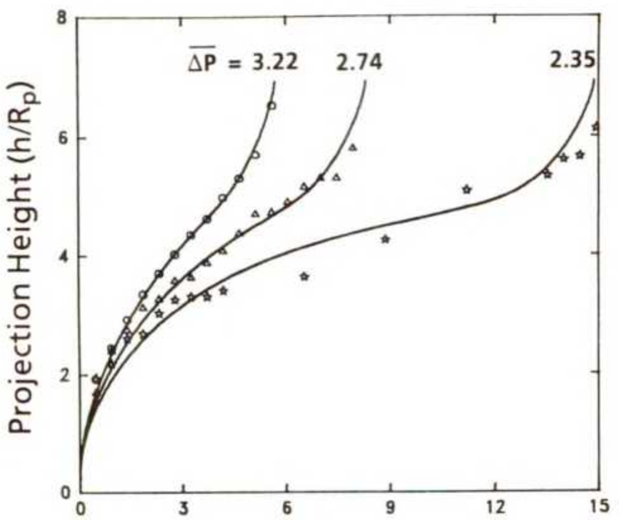

Fig 6. Three curves show the time course of the dimensionless projection height, h/R_p, at three different pressures. Solid lines show the theoretical prediction for a dimensionless cell radius, R_{Do}/R_p, of 5.0 and dimensionless pressures (from left to right), $\overline{\Delta P} = 3.22, 2.74$, and 2.35. Points represent data taken from a single cell at three different pressures: for the circles, $\overline{\Delta P} = 3.44$; for the triangles, $\overline{\Delta P} = 2.74$; and for the stars, $\overline{\Delta P} = 2.35$. The values for the dimensionless pressures for the data represent measured values. The values given for the curves were chosen to give the best agreement between the data and the theoretical curve. The 7.0% difference in the value of $\overline{\Delta P}$ for the circle data and the corresponding curve could have been due to measurement error or frictional interactions. The data were shifted vertically to allow for uncertainty in determining the zero height. The magnitudes of the shifts were 0.70 (circles), 0.54 (triangles), and 0.48 (stars). These corrections were all less than the thickness of the wafer.

RESULTS

The pore used in the present experiments was approximately 1.6 μm in diameter and 1.2 μm in thickness, as measured in the light microscope. A cell in the process of passing through the pore is shown in Fig 5. A total of 28 cells were tested. Six of these were discarded because they tended to adhere to the silicon pore and their passage times were artifactually long. The data from the remaining cells agreed well with the analytic predictions, both for the height of the projection as a function of time and for the total time as a function of the pressure difference across the pore. The sequence in which the different pressures were applied was not ordered. When a pressure was repeated for a given cell, the egress times were similar, ie, there appeared to be no history dependence of the pressure-time relationship.

Three examples of the height of the projection as a function of time are shown in Fig 6. The data were obtained from recordings of a single cell passing through the pore three different times, each time at a different pressure (0.62 mm Hg, 0.72 mm Hg, and 0.90 mm Hg). The solid lines show the theoretical prediction (Equations 3 and 4) for the projection height as a function of time for a cell with an initial radius of 4.0 μm passing through a pore with a radius of 0.8 μm at pressures of 0.62 mm Hg, 0.72 mm Hg, and 0.84 mm Hg.* Because of the finite thickness of the wafer and uncertainty about the exact location of the point, h = 0, each of the data sets was shifted vertically to minimize the average vertical distance between the data points and the corresponding curve. No other adjustments in the data or the theoretical parameters were made.

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*The 7.0% reduction in the value for ΔP for the third curve (compared to the measured value) was chosen because better agreement between theory and data was obtained. This difference can be attributed either to measurement uncertainty or to frictional interactions between the cell and the pore.

It should be noted that not all of the measured time courses of egress agreed as well with the analytic prediction. At pressures near the threshold pressure (the minimum pressure needed to push the cell through the pore) small errors in the values chosen for R_{D_o} or ΔP resulted in large differences in the predicted time course of egress. At high pressures, the accuracy of the measurements made from the videorecordings was limited by the framing speed of the camera, and it was not possible to evaluate the accuracy of the analytic prediction. At intermediate pressures, however, the agreement between theory and observation shown in Fig 6 was typical, although in some cases the values for R_{D_o} or ΔP had to be adjusted to obtain the best fit. Considering the simplicity of the analytic model, the agreement between theory and experiment was remarkably good.

The egress times for 22 different cells over a range of pressures are shown in Fig 7. The theoretical curves are calculated for a pore radius of 0.8 μm and initial cell radii (from left to right) of 3.2 μm, 4.0 μm, and 4.8 μm. For this curve, 14 dimensionless time units correspond to one second, and one dimensionless pressure unit is 350 dyn/cm^2 (0.26 mm Hg). No parameters were used to adjust the data to fit the theoretical prediction. The agreement is again quite good, especially at the larger pressures. At very low pressures, the cells tended to pass through the pore more quickly than expected, and the actual threshold pressure was slightly lower than was predicted.

DISCUSSION

The experimental results confirm the major theoretical predictions. First, there is a threshold pressure on the order of 0.5 mm Hg required to push the cell through the pore, and second, for pressures greater than 1.0 mm Hg, passage times are typically less than one second. The accurate prediction of the change in the height of the spherical projection with time also supports the validity of the analysis and its underlying assumptions.

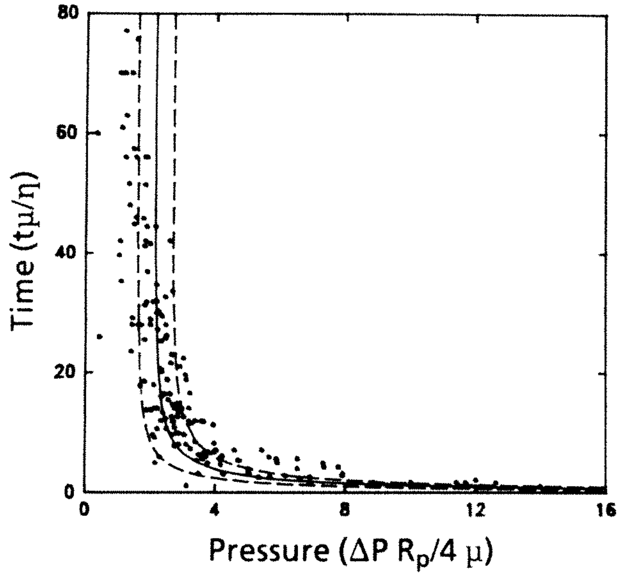

Fig 7. Egress time as a function of pressure. The dots represent all of the data taken for the 22 nonadherent cells tested. The solid curve represents the theoretical prediction for $\overline{R_{D_o}} = 5.0$; the dashed curve at the left is the prediction for $\overline{R_{D_o}} = 4.0$; the dashed curve at the right is the prediction for $\overline{R_{D_o}} = 6.0$. There were no adjustments made to obtain agreement between the theoretical curves and the experimental data.

One slight, but potentially important, difference between the analytic prediction and the experimental results is the lower-than-expected threshold pressure that was observed in the experiments. This difference is most probably due to the cell folding as it passes through the aperture. Such folding would reduce the amount of surface deformation required for passage compared to the deformation of the analytic model. It is important to recognize that because of their ability to fold, real cells can negotiate an aperture at pressures lower than that predicted by the theoretical model.

The slightly higher-than-expected passage times that were observed for pressures between 1.0 and 2.0 mm Hg are probably due to adhesive or frictional interactions between the cells and pore. Cells that took longer than expected to complete their passage generally did not follow the predicted dependence of the projection height on time. The differences between the predicted and the observed time courses were consistent with small frictional or adhesive interactions between the cell and the pore.

Constraints of surface area and volume. The present analysis does not take into account restriction on passage resulting from the conditions of constant cell surface area and volume. Although the present analysis is sufficiently accurate to predict cell passage times when the surface-to-volume ratio is sufficient for cell passage, the constraints of constant area and volume limit the size of an aperture that a cell can negotiate. The strength of this limitation is illustrated by the experimental results shown in Fig 8. A single cell was transferred with a micropipette into a series of chambers with successively decreasing salt concentrations. The pressure needed to aspirate the cell into the pipette (P_t) was measured in each chamber. Over the range of concentrations between 260 mOsm and 230 mOsm only a slight increase in P_t occurred. This slight increase was probably due to a decrease in the ability of the cell to fold, because more and more membrane area was being taken up to encompass the cell volume. The abrupt change in P_t at around 225 mOsm occurred because at this salt concentration the surface area of the cell was not sufficient to enclose the cell volume within the constraint of the pipette geometry.

Whether or not a cell is restricted from passage through a particular pore depends on the surface and volume of the cell and the size and shape of the aperture. In the Appendix a relationship is derived to calculate the minimum surface area a cell with a given volume must have to negotiate a cylindrical pore of given dimensions. In Fig 9, this minimum area is shown as a function of cell volume for three different-sized pores. The solid curve corresponds to a pore of radius (R_p) 0.8 μm and thickness (L) 1.2 μm. These are the measured dimensions of the pore used in the present study. The short-dashed curve corresponds to $R_p = 0.8$ μm and $L = 0.0$ μm. This relationship would be appropriate for the present study if the pore was hour-glass shaped, such that the thickness of the wafer at the aperture was less than the

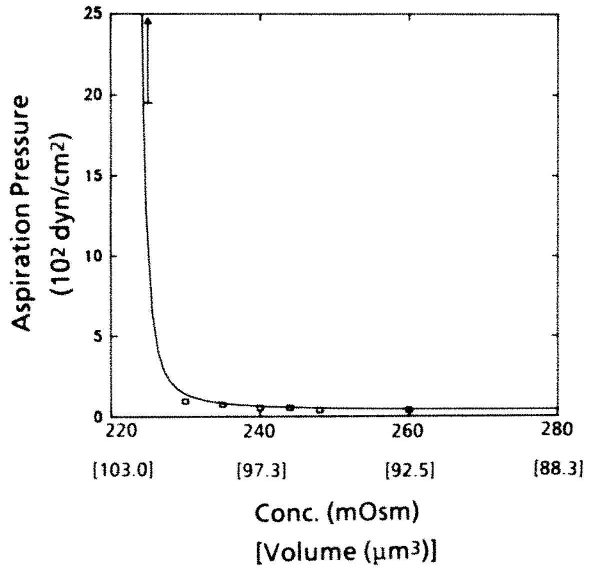

Fig 8. The pressure (P_t) required to pull a cell into a micropipette (3.5 μm ID) is shown as a function of the osmolarity of the suspending solution. Points represent measured aspiration pressures for the same cell into the same pipette in solutions of different salt concentrations. The solid curve is "hand-drawn" and has no theoretical significance. The abrupt change in P_t at around 225 mOsm occurs because the volume of the cell at this concentration is too large to be contained by the cell membrane within the constraint of the pipette geometry. The volumes shown in parenthesis were calculated based on a volume of 100 μm^3 at 230 mOsm assuming that the cell behaved as an osmometer with a nonideality coefficient of 0.6: $[(V - V_i)/V_i = 0.6\ (C_i - C)/C]$, where V_i is the isotonic volume (86.5 μm^3) and $C_i = 290$ mOsm.

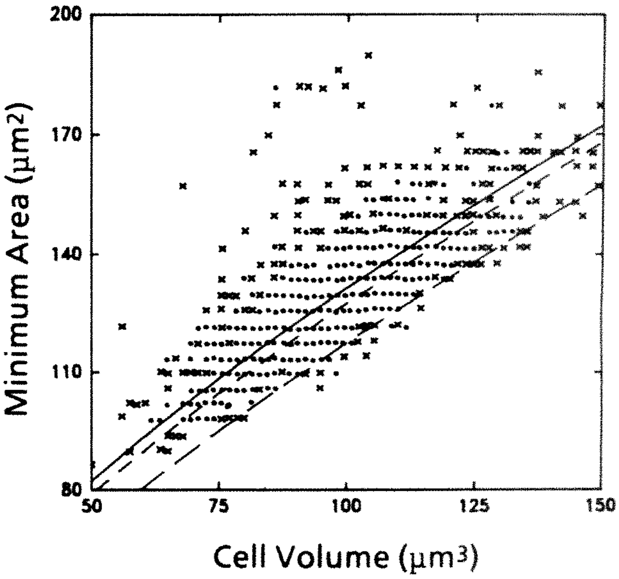

Fig 9. Minimum area required for cell passage as a function of cell volume. The solid line shows the calculated minimum area for the aperture used in the present experiments ($R_p = 0.8$ μm, $L = 1.2$ μm). The short dashed line corresponds to an aperture with $R_p = 0.8$ and $L = 0.0$. The long dashed line corresponds to $R_p = 1.6$, $L = 0.0$. The dots and X's show the range of surface areas and volumes for normal erythrocytes as measured by Tsang.[7] Each X marks a region containing an area and volume of one cell; dots indicate that more than one cell lies in that region.

thickness of the wafer at its edge. The long-dashed curve corresponds to $R_p = 1.6$ μm and $L = 0.0$ μm, a pore twice as large as the one used in the present study. During our experiments no cells were found that were incapable of negotiating the pore, indicating that all of the cells in our study had surface areas and volumes that would place them above the solid (or short-dashed) curve in Fig 9.

This observation is at odds with expectations based on the data of Tsang,[7] who used high-resolution interference holograms to measure the surface area and volume of over 1,500 cells from a variety of donors (see also Fung et al[8]). Tsang's data are shown as the shaded region in Fig 9. A significant fraction of the cells in Tsang's study lie below the minimum area curves for the pore size used in our study. Cells falling below the curve for a particular aperture should be incapable of passing through, except under very large pressures or after lysis. If the surface areas and volumes of the cells used in our study had the same surface area and volume distribution as the cells in Tsang's study, we would have expected a significant fraction of them to be unable to pass through the pore under the pressures used in our experiments. The fact that all of the cells in our study passed through was surprising.

The reason for this discrepancy is not clear. It is unlikely that it is due to an error in measuring the pore diameter, because the pore would have to have a diameter twice as large as was measured to accommodate most of the cells in Tsang's study (note the long-dashed line in Fig 9). Other explanations seem more plausible. Differences in the suspending buffers could account for at least part of the difference. (Tsang used a TRIS buffer whereas we used phosphate.) Additionally, there may have been unconscious bias in cell selection for our measurements. An earlier, less extensive study by Evans[9] using the same technique as Tsang found volumes 4.0% smaller and surface areas 4.0% larger than those reported by Tsang. This difference was thought to be due to bias in cell selection. The discrepancy between our observations and Tsang's results could be accounted for by such small differences.

Clearly, when the area of the cell is insufficient to encompass the cell volume during passage, the present analysis is inapplicable. However, as the data in Fig 8 show, when the area is sufficient, the cell volume has little effect on the dynamics of cell passage.

Implications about bone marrow egress. The results of these experiments support the feasibility of pressure-driven egress of reticulocytes from the bone marrow. Although leukocytes clearly have the capability to move themselves across an endothelial boundary, the mechanism by which reticulocytes pass into the marrow sinuses has remained obscure. Circulating reticulocytes show no evidence of intrinsic motility. If reticulocyte egress is pressure-driven, a major question remains as to the source of the pressure gradient across the pore. Two features of the present results bear on this question. The first is that the pressure required to force a cell through an aperture is small compared to systemic pressures. Michelsen[10] has measured pressure differences of 20.0 mm Hg between the marrow tissue and the emissary vein just outside the cortical bone in rabbits. It is reasonable to hypothesize that a part of that pressure drop sufficient to drive reticulocyte egress (2.0 to 4.0 mm Hg) might exist across the pore between the hematopoietic space and the marrow sinus, which is contiguous with the emissary vein.

The second feature of the present results that bears on the question of the origin of the driving pressure is the short time required to complete egress (~0.3 s) when the threshold pressure is exceeded. The pressures that Michelsen recorded were pulsatile,[10] and it is likely that pressures across the pore sufficient to cause egress, occur only transiently. Dabrowski et al[11] have shown a correlation between transient increases in marrow pressure and an increase in the release of reticulocytes into the circulation in rabbits. The fact that the times for cell passage that we have measured are less than, or on the order of, the heart rate is an important observation because it shows that if sufficient pressures for egress occur transiently with the pulse, there would still be sufficient time for egress to be completed.

It is important to recognize that in the present study we have used mature erythrocytes to develop and test our theories. If the membrane elastic modulus or the membrane viscosity of early reticulocytes were significantly different from mature cells, our conclusions might be affected. However, preliminary experiments in our laboratory on blood cells of the rabbit indicate that differences in membrane properties between mature erythrocytes and reticulocytes are slight. The average value measured for reticulocyte shear modulus of 5.5×10^{-3} dyn/cm was similar to the control value, 4.5×10^{-3} dyn/cm, and the viscoelastic recovery time constants (the ratios of the membrane viscosity to the membrane shear modulus) were also similar: 0.074 seconds for reticulocytes and 0.081 seconds for controls. The reticulocyte samples in those experiments were prepared by density gradient centrifugation. More than 90% of the cells in those samples were reticulocytes as determined by New Methylene Blue staining. However, in our experiments we selected against oddly shaped cells in favor of those that were biconcave and our data sample probably included more late than early reticulocytes. Nevertheless, it is unlikely that the properties of even very young reticulocytes are so different from mature cells that the conclusions of the present work would be affected substantially.

Our ability to critically evaluate these ideas is hampered by a lack of information about the local environment within the marrow. We have had to use pore dimensions measured in the mouse, pressure differences measured in the rabbit, and transit times and threshold pressures measured on human erythrocytes. Although differences in the architecture and hydrodynamics found within different animals may affect the details of the regulation process for each animal, the major conclusions of the present study should not be affected by anatomic differences between different species. The pressures required to move red cells across thin circular apertures are within the physiologic range, and the speed with which the passage is completed is sufficient for it to occur within the space of a single heartbeat.

ACKNOWLEDGMENT

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APPENDIX

Because erythrocytes maintain essentially constant surface area and volume,[12,13] there are limits on the size and shape of an aperture that a cell can negotiate. In this appendix, a functional relationship is developed that makes it possible to calculate the maximum volume that a cell of a given surface area can have and still be able to pass through a cylindrical pore of given dimensions.

To begin the calculations, consider the shape shown in Fig 10. The surface area, A, and volume, V, of this surface of revolution are:

$$f_A = A = 2\pi\{R_1(R_1 + \sqrt{R_1^2 - R_p^2}) + R_2(R_2 + \sqrt{R_2^2 - R_p^2}) + R_pL\} \quad (A1)$$

$$f_v = V = {}^2\!/_3\pi\{R_1^3 + (R_1^2 + R_p^2/2)\sqrt{R_1^2 - R_p^2} + R_2^3 + (R_2^2 + R_p^2/2)\sqrt{R_2^2 - R_p^2} + {}^3\!/_2R_p^2L\}. \quad (A2)$$

For a fixed volume, we wish to find the relationship between R_1 and R_2 that requires the largest surface area. This is the "critical geometry" for cell passage, ie, if the cell can assume that geometry it will be able to negotiate the aperture. We use the method of Lagrange multipliers and define the function:

$$H = f_A - \lambda(V - f_v). \quad (A3)$$

where λ is the Lagrange multiplier. At the extremum three conditions must be met:

$$\frac{\partial H}{\partial R_1} = 0; \frac{\partial H}{\partial R_2} = 0; \frac{\partial H}{\partial \lambda} = 0. \quad (A4)$$

The third condition simply gives the expression for the cell volume (Equation A2). The first two conditions require that:

$$4R_1(R_1 + \sqrt{R_1^2 - R_p^2}) - 2R_p^2 + \lambda[2R_1^2(R_1 + \sqrt{R_1^2 - R_p^2}) - R_1R_p^2] = 0 \quad (A5)$$

and,

$$4R_2(R_2 + \sqrt{R_2^2 - R_p^2}) - 2R_p^2 + \lambda[2R_2^2(R_2 + \sqrt{R_2^2 - R_p^2}) - R_2R_p^2] = 0. \quad (A6)$$

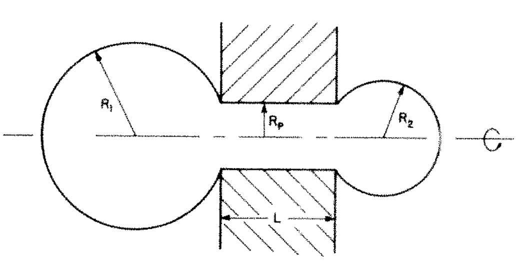

Fig 10. Schematic of a cell caught in a cylindrical aperture. The length of the aperture is L and its radius is R_p. The contours of the cell on either side of the aperture are spherical with radii R_1 and R_2.

After solving Equation A5 for λ and substituting that expression into Equation A6, it can be seen by inspection that both conditions will be met when $R_1 = R_2$. Let R_o be the outer radius when the two outer radii are equal. Then the expressions for area and volume at the critical geometry are:

$$A = 4\pi R_o (R_o + \sqrt{R_o^2 - R_p^2}) + 2\pi R_p L \qquad (A7)$$

$$V = \tfrac{4}{3}\pi[R_o^3 + (R_o^2 + R_p^2/2)\sqrt{R_o^2 - R_p^2}] + \pi R_p^2 L. \qquad (A8)$$

Equation A7 can be solved for R_o:

$$R_o = C_1 (2C_1 - R_p^2)^{-1/2} \qquad (A9)$$

where $C_1 = A/4\pi - R_p L/2$. Equation A9 can be substituted into Equation A8 to eliminate R_o and obtain a functional relationship among A, V, R_p, and L:

$$f(A, V, R_p, L) = 0 \qquad (A10)$$

To generate the curve in Fig 9, the pore dimensions R_p and L were specified, and an arbitrary value for A was chosen. R_o could then be calculated via Equation A9, and the volume, V, corresponding to the chosen value of A could be calculated directly from Equation A8. This volume is the maximum that a cell of area A can have and be able to pass through a pore of the given dimensions. Equation A10 can also be used to calculate permissible pore dimensions for cells with specified surface area and volume, but numerical techniques are required to obtain such values.

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Purification of Murine Erythropoietin Produced in Serum-Free Cultures of Erythroleukemia Cells

By R.-L. Qian, K. Chin, J.-K. Kim, H.-M. Chin, J. Cone, and W.D. Hankins

We previously documented that several erythroleukemia cell lines released factors that stimulated erythropoiesis in vivo and in vitro. A simple five-step scheme has been devised that allows purification of this erythropoietic activity to apparent homogeneity. The methods employed included lectin affinity chromatography (wheat germ agglutinin), gel filtration (ultro gel ACA44), ion exchange, hydroxylapatite, and high performance liquid chromatography. Following polyacrylamide gel electrophoresis, biologic activity was recovered in an area corresponding to a molecular weight of 35,000 daltons. Silver staining of a polyacrylamide gel after electrophoresis of our most purified preparation revealed a single band at 35,000 daltons.
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WE previously reported[1,2] that erythroleukemia cells derived from mice infected with Friend murine leukemia virus (F-MuLV) produced erythropoietic factors with properties similar to sheep and human erythropoietin (Epo). Subsequent studies showed that the murine Epo was active in nine separate in vivo and in vitro erythropoietic assays, and was neutralized by antibodies prepared against partially purified human Epo. While not all erythroid lines isolated from Friend virus-infected mice were positive for Epo production, four independently isolated lines made significant quantities of the hormone. We also found that Epo production by these cells continued in the absence of serum.[2] This finding has permitted us to devise a relatively simple purification scheme for the murine Epo. A five-step procedure has produced an apparently homogeneous preparation of murine erythropoietin that is active in vivo and in vitro, and has an apparent molecular weight of 35,000 daltons.

MATERIALS AND METHODS

Chromatographic materials were obtained commercially as follows: wheat germ agglutinin (Pharmacia, Uppsala, Sweden); ACA44 (LKB Instruments, Inc, Gaithersburg, Md); hydroxylapatite, fast flow (Calbiochem, San Diego, Calif). The Mono Q anion exchange resin was a part of Pharmacia's Fast Liquid Chromatography System (Uppsala, Sweden). High pressure liquid chromatography was performed on an LC-8-D8 Supelco column (15 cm × 4.6 mm).

Erythropoietin assays. Following purification by hydroxylapatite chromatography, erythropoietic activity of Epo was verified by its ability to stimulate ^{59}Fe incorporation in the polycythemic mouse assay.[3] At all other times, dose-dependent stimulation of tritiated thymidine (^{3}H-Tdr) by spleen cells from phenylhydrazine-treated mice was regarded as an index of Epo activity. Assay conditions were exactly as described by Krystal.[5]

Erythroleukemia cultures. We previously reported that several erythroleukemia lines secreted Epo.[2] The line used for the present purification of Epo was HFTC-5. HFTC-5 cells were seeded in 50 mL of medium in 150 cm Falcon flasks at two million cells/mL. The medium used was Iscove's modified Dulbecco's medium supplemented with 100 μg/mL transferrin and 100 μg/mL insulin. Cultures were incubated for 10 to 14 days and the conditioned medium monitored for Epo content with the ^{3}H-tdr assay. When the Epo level reached greater than 1 U/mL, the cells were removed by centrifugation (2,000 rpm for 5 min), and heat treated at 56 °C for one hour to inactivate the virus produced by the erythroleukemia cells. Conditioned medium was stored at 4 °C for up to one month without loss of activity.

RESULTS

Lectin chromatography. 2,000 mL of conditioned medium was applied directly to wheat germ agglutinin[4] column (2.5 cm × 27 cm; bed volume was about 100 mL) at a flow rate of 24 mL/hr. When loading was completed, the column was washed with 300 mL of PBS, following which the Epo was eluted with PBS containing N-acetyl glucosamine (0.5 mol/L). The activity and optical density (OD) profile is shown in Fig 1. Routinely, greater than 50% of the activity was recovered in a peak that trailed the second optical density peak. Specific activities for the WGA-purified material was around 27.6 U/A. The peak fractions were pooled, dialyzed against water, lyophilized, and stored at −70 °C. This material was referred to as Step I Epo. In other experiments, Epo was eluted with a gradient of N-acetylglucosamine (not shown). Neither the recovery nor the purity of Epo was improved with the gradient elution.

Gel filtration. Lyophilized Step I Epo was dissolved in 5 mL PBS (PH 7.4) and loaded onto an ACA44 gel filtration column with dimensions of 2.5 cm × 84 cm and a void volume of 130 mL. The column had been standardized with albumin (66,000 d), ovalbumin (43,500 d), chymotrypsin (25,000 d), and ribonuclease A (13,700 d). As shown in Fig 2, Epo activity was recovered between the ovalbumin and chymotrypsin peaks. The average molecular weight of the Epo peak was 35,000 to 36,000. The Epo peak was divided into three fractions referred to as head (tubes 46 and 47), middle (tubes 48–57), and tail (tubes 58–61). By pooling these three fractions, the recovery of Epo from gel filtration was in excess of 90%, a specific activity of 316.52 U/A. However, subsequent chromatographic procedures and PAGE analysis revealed that by saving only the middle portion, a high molecular protein of 66,000 (probably albumin) was virtually eliminated as a contaminant. The specific activity in this pool was 652.92 U/A (Table 1) and the recovery was 70.2%. This material was referred to as Step II Epo and was dialyzed against water, lyophilized, and stored at −70 °C.

Ion exchange chromatography. 100 to 200 units of Step II material was dissolved in water and loaded onto a mono Q anion column of Pharmacia's Fast Performance Liquid

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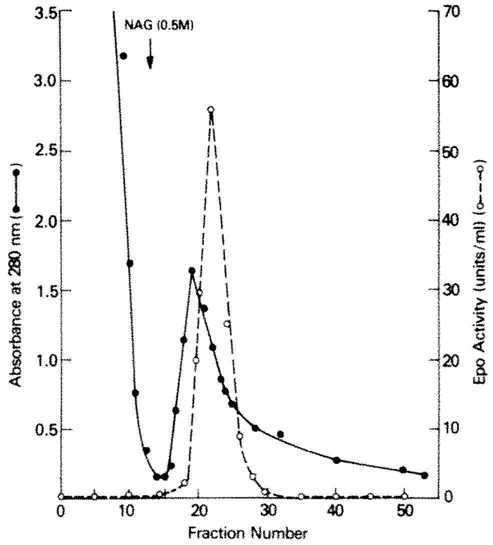

Fig 1. Affinity chromatography of erythropoietin. The serum-free supernatant of HFTC-5 cell line was applied to wheat germ agglutinin column (2.5 cm × 27 cm). Flow rate: 24 mL/hr.

Chromatography instrument. Preliminary analytical runs employing a continuous linear gradient of NaCl revealed that Epo was eluted between 0.14 mol/L NaCl to 0.25 mol/L NaCl with a peak at 0.18 mol/L NaCl. Therefore, in this and subsequent runs, the proteins were eluted with a discontinuous gradient of NaCl in a 0.01 mol/L Tris-HCl buffer (PH 7.5), as shown in Fig 3. Epo was eluted in two unequal peaks. Less than 5% of the erythropoietic activity was found in the small peak (0.1 mol/L NaCl). This material was not further analyzed. Greater than 70% of the activity was routinely recovered in 0.3 mol/L NaCl, and had a specific activity of 1,112.50 U/A. This material was dialyzed against water, lyophilized, and stored at −70 °C as Step III Epo.

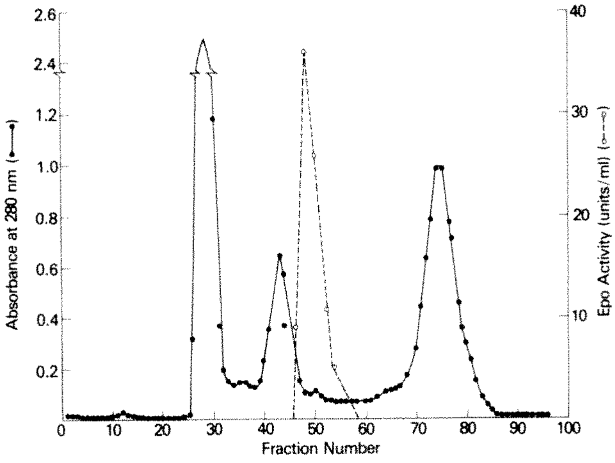

Fig 2. Gel filtration of Step I Epo on Ac44 column (84 cm × 2.5 cm). Flow rate: 5.5 mL/30 min. The column was standardized empirically using commercial molecular weight standards. The peaks albumin (67,000 d), ovalbumin (43,000 d), chymotrypsin (25,000 d), and ribonuclease (13,700 d) were recovered in fractions numbers 42, 52, 65, and 82, respectively.

Table 1. Summary of Purification of Murine EPO

Procedure	Absorbance	EPO Units	Spec Act	Fold Purified
None	11.6×10^3	2,512	0.21	—
WGA	46	1,274	27.6	131
Gel filtration	1.37	894	653	3,109
FPLC	0.56	623	1112	5,297
Hydroxylapatite	0.05	225	4500	21,000
HPLC	—*	130	—	—

Absorbance was determined at 280 mu and erythropoeitic activity was assessed by a ^{3}H-tdr uptake assay. Spec Act refers to specific activity expressed as Units of Epo/A_{280}.

*Although tested, no absorbance was detected in the HPLC-purified EPO.

Hydroxylapatite chromatography. 300 to 600 units of Step III material was added to a hydroxylapatite[6] column that had been equilibrated in 0.02 mol/L phosphate buffer, PH 6.8. A gradient of increasing phosphate concentration (0.02 to 0.1 mol/L) was employed to elute the proteins. As shown in Fig 4, three OD peaks were observed. The Epo activity profile was a symmetrical peak that overlapped the second major protein peak. The second small peak of Epo activity at fraction 29 was not reproducible and was, therefore, not included in the pooled fractions. Fractions were pooled, dialyzed, lyophilized, and stored at −70 °C as Step IV Epo. The recovery of activity from this procedure ranged between 50% and 70%.

Neuraminidase treatment and electrophoresis. To characterize the Epo purified to this stage, neuraminidase (0.06 units) was added to a solution of 100 units of Step IV Epo and dissolved in 25 μL of 50 mmol/L sodium acetate buffer, PH 5.2. This mixture was incubated at 37 °C for three hours and then boiled to destroy the neuraminidase. This material was combined with electrophoresis sample buffer, boiled for three minutes, and loaded onto a 10% polyacrylamide gel. A control preparation of Step IV Epo was similarly treated with omission of the neuraminidase. The samples were electrophoresed for four hours at a constant current of 15 mA, after which the gels were cut into 50 horizontal slices (2 mm/slice). Each slice was placed in a 12 × 75 mm glass tube to which was added 1.0 mL PBS, PH 7.4. The gel slices were mashed extensively and allowed to stand overnight at 37 °C. The following morning, the gel matrix was removed by

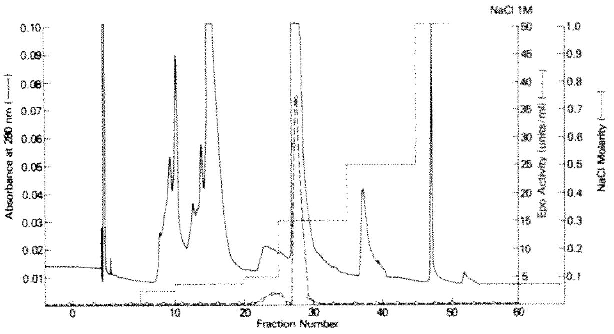

Fig 3. Ion exchange chromatography of Step II Epo on a mono Q column (5.5 cm × 1 cm). Flow rate: 1 mL/min.

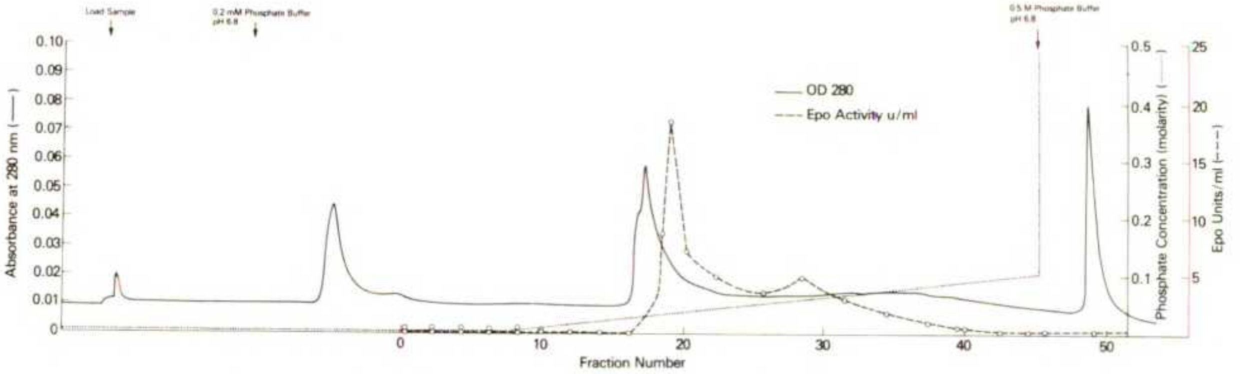

Fig 4. Purification of Step II Epo with hydroxylapatite column (1.5 cm × 5 cm). Flow rate: 2 mL/min.

centrifugation and the supernatant assayed for Epo activity. The activity profile is shown in Fig 5A. The fractions containing Epo activity were pooled, dialyzed, and again subjected to polyacrylamide gel electrophoresis (PAGE). The results, shown in Fig 5b, demonstrate that the biologic activity comigrates with a single band of silver stained material in the molecular weight range of 35,000 daltons. Yanagawa et al[7] observed that neuraminidase treatment of human Epo yielded molecules that migrated faster than nontreated Epo. As seen in Fig 5, neuraminidase-treated murine Epo also migrated slightly ahead of nontreated Epo.

High performance liquid chromatography. 200 units of Step IV material was dissolved in 0.1% TFA and applied to a Supelco LC-8-DB HPLC column. Protein was eluted with a gradient of 30% to 60% acetonitrile in 0.1% trifluoroacetic acid. More than 50% of the activity was recovered in fractions 66–68 (Fig 6). When this material was subjected to PAGE and silver stained (Fig 7), a single band of protein was recovered in a region that corresponded to 35,000 daltons. It should be noted that this gel was "overstained" in order to visualize the Epo bands. Even so, no other proteins were detected although some artifactual staining was observed in

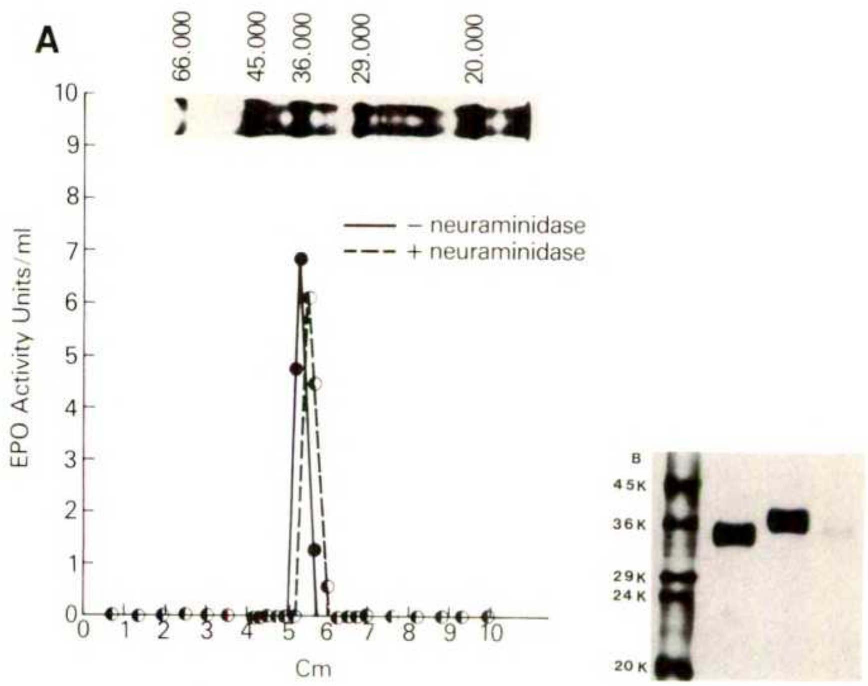

Fig 5. (A) SDS-polyacrylamide gel electrophoresis and erythropoietin step IV before (solid line) and after (dashed line) neuraminidase treatment. The slices containing maximum erythropoietic activity were eluted, pooled, dialysed, concentrated, and applied to a second SDS-polyacrylamide gel electrophoresis. In addition, Lane 1. Standards: albumin, egg, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,100. Lane 2. Approximately 20 units of neuraminidase treated Step IV murine Epo recovered from 6A in the area from 5.0 to 5.4 cm. Lane 3. Approximately 20 units of untreated Step IV Epo recovered from 6A in the area from 5.4 to 5.8 cm. Lane 4. Six units of purified human EPO (Toyoba Inc, Japan). Following electrophoresis the gel was stained with silver.

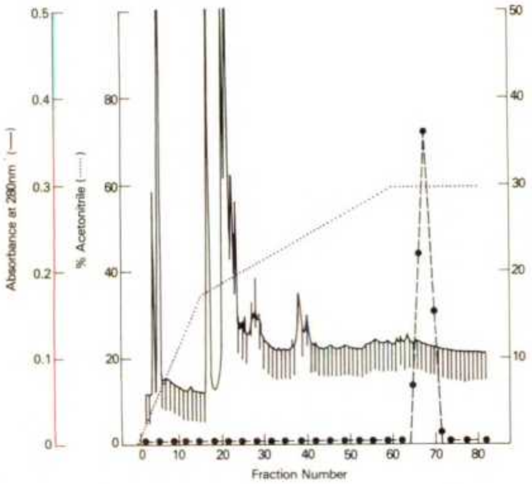

Fig 6. High performance liquid chromatography of Step IV Epo. 200 units of Step IV Epo was applied to HPLC column (4.6 mm × 150 mm). Flow rate: 1 mL/min.

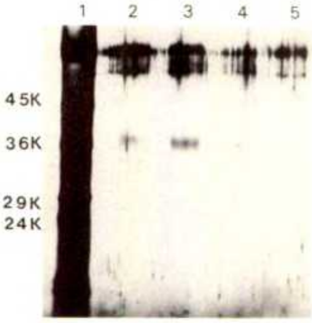

Fig 7. SDS-polyacrylamide gel electrophoresis of HPLC purified Epo. Lane 1. Standards: albumin, egg, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000. Lane 2. Step V Epo (Tube no. 65 & 66), ~10 units. Lane 3. Step V Epo (Tube no. 67 & 68), ~30 units. Lane 4. Step V Epo (Tube no. 69 & 70), ~6 units. Lane 5. Sample buffer containing only the marker dye.

the high molecular weight area in all lanes, including the control (lane 5), which received only the marker dye.

DISCUSSION

Discovery of multiple erythroleukemia lines that make erythropoietic growth factors provided both an opportunity and a challenge for our laboratory. First, a stable cell culture source of murine Epo has long been needed for research purposes. Second, since our findings represented the first observation of Epo production by erythroid cells, conceptual questions arose concerning the relation of Epo production to leukemic pathophysiology as well as normal red cell development. Finally, we felt that a detailed analysis of this murine erythropoietic factor might provide further insight into erythropoietin structure and function. We therefore undertook to devise a rapid and simple method, using conventional procedures that would permit isolation of homogeneous murine erythropoietin. As starting material, we used supernatants from erythroleukemia cells cultured in the absence of fetal calf serum for 10 to 14 days. Such conditioned media contained the equivalent of at least 1 U/mL of erythropoietin. The starting specific activity was 0.21 U/mg. Sequential chromatography of this material through columns of agarose-bound wheat germ agglutinin, ACA44 gel filtration matrix, ion exchange resin, hydroxylapatite, and high performance liquid chromatography has yielded a protein with erythropoietic activity in vivo and in vitro, and which produced a single band upon polyacrylamide gel electrophoresis. The apparent molecular weight of the electrophoretic band was 35,000 daltons. The overall recovery of erythropoietic activity through all the procedures was 5% to 15%. A summary of a typical purification is shown in Table 1.

Both human and sheep erythropoietin have been reported to be glycoproteins.[4,6-8] Our finding that neuraminidase treatment of hydroxylapatite purified material increased the electrophoretic mobility of the erythropoietic activity and abolished the in vivo activity[2] are consistent with the interpretation that murine erythropoietin is also a glycoprotein.

The data in this report provide further support for our contention that the eythropoietic factors produced by the erythroleukemic lines are indeed erythropoietin.[1,2] Since human Epo is the only erythroid regulatory factor that has been purified, sequenced, and molecularly cloned,[8] it seems an appropriate molecule for comparison. Our previous results[1,2] and those of Choppin et al[3] demonstrated many similarities between human Epo and the factors produced by erythroleukemia cells. For example, all the physical and chemical properties assessed for murine and human Epo have been identical.[1-3] Furthermore, we demonstrated immunologic cross-reactivity between murine Epo and antisera raised against human Epo.[2] Since the reported molecular weight of human Epo is approximately 35,000[6] we can now include molecular weight amongst the similarities between murine and human Epo. Nevertheless, for a complete comparative analysis, the amino acid sequence and the nucleic acid sequence of the corresponding genes will be necessary. Experiments to derive this additional information are presently underway.

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Erythropoietin, an Autocrine Regulator? Serum-Free Production of Erythropoietin by Cloned Erythroid Cell Lines

By W. David Hankins, John Schooley, and Christina Eastment

Production of lymphoid and myeloid growth regulatory factors by hematopoietic cells is well documented. On the other hand, the major site of production of erythropoietin (Epo), which regulates physiologic red blood cell development, is thought to be the kidney. Here we report the isolation of multiple erythroleukemia cell lines that produce erythropoietic factors and present extensive biological, immunologic, and biochemical evidence to document that the active agent is Epo. The erythropoietic activity was neutralized by Epo antiserum and exhibited physical properties indistinguishable from those of human and sheep Epo. Positive lines produced between 0.1 and 1.5 U/mL of Epo, which stimulated erythropoiesis in vivo and in vitro in nine biological assays. Twenty sublines derived from single cells were inducible for hemoglobin and spectrin synthesis. All the sublines produced Epo. Production of the hormone continued when the cells were seeded in the absence of serum. Our finding that multiple independent isolates produce Epo raises the possibility that Epo production by erythroid precursors may play a role in normal erythropoiesis or, alternatively, that Epo gene activation may be a relatively common occurrence that contributes to, or is associated with, certain forms of virus-induced leukemias.

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GROWTH AND DIFFERENTIATION of blood-forming elements are influenced by a family of diverse molecules, referred to collectively as hematopoietic regulators. Although their in vivo roles are poorly defined, these regulators clearly have proliferative or differentiative actions on specific target cell populations in the various hematopoietic lineages. Although produced in vitro by a variety of cell types, the in vivo site of synthesis for most hematopoietic regulators is unknown. Erythropoietin (Epo), a glycoprotein hormone, appears to be the exception to these statements, since numerous in vivo and in vitro experiments have demonstrated that an in vivo role of this hormone is in the regulation of red cell development.[1,2] Furthermore, while limited in vitro production of Epo has been observed, it is generally held that the major in vivo site of production of this hormone is the kidney, although extrarenal production has been documented.

During recent attempts to isolate new erythroleukemia lines from Friend virus-infected mice, we made several observations that led us to suspect that Epo might be produced by erythroid cells. First, transplantable erythroleukemia cells grew better at high density than at low density, and addition of Epo or conditioned media from an established erythroleukemia line stimulated growth of these primary cultures.[3] Second, the conditioned medium also stimulated hemoglobinization of erythroid bursts induced in vitro by the anemia-inducing strain of Friend virus complex. Third, when tested for tumorigenicity in nude mice, IW32 erythroleukemia cells[4] induced a polycythemia concomitant with tumor formation (D. Hankins and P. Tambourin [Pasteur Institute, Paris], unpublished observations, December 1982).

We therefore isolated and screened numerous erythroleukemia lines for production of erythroid growth factors. We report here the identification of three erythroid cell lines that secrete erythropoietic factors that are active in vivo and in vitro and have biochemical, immunologic, and physical properties identical to those of erythropoietin. Clones of Epo-producing cells were derived, characterized as erythroid cells, and shown to produce Epo in the absence of serum. A companion paper in this issue describes purification of this murine Epo to apparent homogeneity.

MATERIALS AND METHODS

Derivation of erythroleukemia lines. H-11 and the uncloned HFTC lines were derived on different occasions from liver or spleen explants prepared from separate Friend murine leukemia virus[5]-infected mice (W.D.H., unpublished observations, March 1980 and February 1981). Procedures used to induce erythroleukemia and establish permanent cell lines were as described by Oliff et al.[6] In an attempt to induce de novo generation of spleen focus-forming virus (SFFV), we exposed HFTC cells to 5-azacytidine as described by Graf et al.[7] The cells that survived (<10%) were cloned and frozen. Several clones were screened for SFFV production and were uniformly negative. Because of subsequent observations, several HFTC clones were retrieved and screened for the production of erythroid growth factors. MELC-FVA-28 and MELC-FVP-16 were established from mice infected with the anemia (FVA) or polycythemia (FVP) strains of Friend virus complex, respectively, as described by Mager et al.[8] TP-3 was originally isolated and characterized as an erythroleukemia line by Oliff et al.[6] IW32, another transplantable erythroleukemia line, was isolated and introduced to our laboratory by Dr Pierre Tambourin as previously described.[4] All transplants were in NFS/N or NIH nu/nu mice, which were obtained from the National Institutes of Health small-animal facility in Bethesda, Md.

Erythropoetic assays. Erythropoietic assays were standardized, using Connaught (Toronto) Step III Epo. CFU-E and BFU-E erythroid colony assays in semisolid media were performed as described.[9-11]

For ^{59}Fe uptake assays, hematopoietic cells were cultured in 0.8% methylcellulose containing 30% fetal calf serum (FCS) for two days, as previously described.[12] Twenty-four hours before harvest, $^{59}FeCl_2$ was added to 1 μCi/mL. After the 24-hour pulse, cells were harvested and the ^{59}Fe incorporated into heme was assessed.[12] The in vivo polycythemic mouse assay was performed as previously described.[13] The tumorigenicity assay was performed by subcutaneous inoculation of NIH nu/nu mice with 0.2 mL of Dulbecco's

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minimum essential medium (D-MEM) containing 5 × 10^6 erythroleukemic cells. Animals were observed during the subsequent four months for tumor formation. Hematocrits were determined in 20 μL microhematocrit tubes (Drummond) on each mouse in which subcutaneous tumors developed. Tritiated thymidine (^{3}H-tdr) incorporation assays were performed as described by Krystal.[14]

Virus assay. The SFFV assay was performed as described by Axelrad and Steeves.[15]

Antibody neutralization of erythropoietin. Preparation of the erythropoietin antiserum used in these experiments was described by Schooley and Garcia.[16] Goat antirabbit IgG was purchased from Cappel Laboratories, Malvern, Penn.

Chemicals and enzymes. Neuraminidase, trypsin, ribonuclease, deoxyribonuclease, and ammonium sulfate were purchased from Calbiochem (San Diego). Treatment of conditioned media with neuraminidase and trypsin was as described previously.[17] To assess nuclease sensitivity of the erythropoietic factors, 1 μg/mL of either ribonuclease or deoxyribonuclease was added to conditioned media and incubated for one hour at 37 °C. In control experiments, similar incubations of these enzymes rendered RNA and DNA (100 μg/mL) completely soluble in 10% perchloric acid after one hour of incubation at 37 °C.

Serum-free culture. HFTC-5 cells were grown in D-MEM containing 10% FCS to a density of 2.0 × 10^6 cells per milliliter. Cells were collected and seeded at 2 × 10^6 cells per milliliter in Iscove's modified D-MEM containing insulin (1 μg/mL) and transferrin (1 μg/mL). Such cultures were incubated for 14 days at 37 °C. After removal of the cells by centrifugation, conditioned media were filtered through a 0.45-μm filter and stored at 4 °C.

Single-cell cloning. The indicated cell lines were seeded in D-MEM + 10% FCS in 96-well microtiter dishes (0.1 mL per well), with each well containing an average of 100, 10, 1, or 0.1 cells per well. After one week of incubation, each well was screened microscopically. Wells containing an estimated 64 to 128 cells were marked as potential single-cell clones, based on an observed doubling time of 18 to 24 hours. After a further week of growth, ten such clones were transferred to 1-mL dishes and expanded. Each of the ten clones was then subjected to another cycle of single-cell cloning, and second-cycle clones were picked and expanded, and tested for Epo production.

RESULTS

Screen of hematopoietic cells for erythroid growth factors. At least three virus preparations have been isolated from the origional virus stocks isolated by Dr Charlotte Friend.[18] Each of these preparations induces a qualitatively distinguishable form of erythroleukemia (for review, see ref. 19). One stock contains only a single replication-competent helper murine leukemia virus (F-MuLV) and appears to induce a rapid erythroleukemia when injected into newborn NIH Swiss mice. Two stocks, FVA and FVP, are complexes containing the anemia- or polycythemia-inducing form of SFFV in addition to F-MuLV. These complexes induce a profound erythroblastosis within two to three weeks after inoculation of either adults or newborns. We and others have isolated a number of cell lines from the erythroleukemias induced by each of these viruses. Due to the preliminary observations described earlier, we screened numerous murine cell lines for erythroid growth factors. In our initial screen we tested the ability of various conditioned media to stimulate hemoglobin synthesis by cultured fetal liver cells.[20] Of 12 FVP lines, nine FVA lines, and nine F-MuLV lines tested, only four produced detectable erythroid growth factors (Table 1). The positive lines, HFTC (subclones 5 and 14), IW32, H11, and TP-3, were all derived from F-MuLV-infected mice.

We next tested media from two human erythroleukemia[23,24] and two chemically induced rat erythroleukemia lines.[22] No erythroid activity was detected in supernatants from any of these lines.

Biological actions of murine erythroleukemia cell conditioned media. The ^{59}Fe assay can be influenced by factors other than Epo,[25] and changes in specific activity of ^{59}Fe could possibly explain the apparent stimulation of hemoglobin. We therefore tested the media for erythropoietic activity in five additional assays in vitro and three different in vivo assays (Table 2). The media from HFTC-5 and TP-3 were active in all nine biological assays. We observed that media from HFTC-5 and TP-3 produced dose-response curves similar to those of Connaught Step III Epo in at least two of the nine assays (results not shown). We conclude that while each of the individual assays may have deficiencies, the collective data from nine erythropoietic assays provide compelling evidence that the biological actions in media from HFTC-5 and TP-3 cultures were identical with those of erythropoietin. Conditioned media from HFTC-5 and TP-3 were tested for interleukin 3 activity, and they were negative (J. Ihle, personal communication, February 1983). Neither media could substitute for Wehi conditioned media as a source of burst-promoting activity (BPA)[11] in a mixed erythroid colony assay (not shown). Finally, TP-3 and

Table 1. Screen of Hematopoietic Cell Lines for Erythroid Growth Factors

Cell Line (reference)	In Vitro Assay (U/mL)	In Vivo Assay (U/mL)
Mouse		
HFTC-5 (Hankins)*	0.50-1.33	0.8-1.50
HFTC-14 (Hankins)*	0.13-0.30	0.08-0.13
HFTC-18 (Hankins)*	None	None
IW32 (Tambourin)[4]	0.52-0.80	0.3-0.5
H-11 (Hankins)*	0.28-0.42	0.12-0.56
TP-3 (Oliff)[6]	0.32-0.51	0.35-0.48
MELC 745 (Friend)[21]	None	ND
MELC FVA-28 (Hankins)*	None	ND
MELC FVP-16 (Hankins)*	None	ND
Rat		
EL-K6 (Maeda)[22]	None	ND
EL-K20 (Maeda)[22]	None	ND
Human		
K562 (Lozzio)[23]	None	ND
EL (Papayannopoulou)[24]	None	ND

Ten microliters of test sample was added to 0.10-mL cultures of 12- to 13-day fetal liver cells[20] and ^{59}Fe incorporation assayed 48 hours later as described.[12] Epo content was estimated by comparison to a standard curve generated with Connaught step III Epo. In our hands, this assay reproducibly detects the equivalent of 10 mU of Epo per 0.1-mL culture. Therefore, media containing more than 100 mU/mL would register positive. ^{59}Fe incorporation determined as described by Filmanowicz and Gurney.[13] The lower limit of detection in this assay is an Epo concentration of 50 mU/mL. ND, not done.

*See Materials and Methods.

Table 2. Erythroid Stimulation by Conditioned Media From HFTC-5 and TP-3

	Form of Data	No Addition	Connaught Epo	HFTC-5	TP-3
In Vitro Assays					
1. FVA Cells[20]	cpm/5 × 10^5 cells	56	4,670	4,350	3,414
2. DNA synthesis[25]	cpm/4 × 10^5 cells	1,128	18,024	32,133	7,851
3. CFU-E[26]	Colonies per 10^6 cells	0	850	885	308
4. 2-day ^{59}Fe[19]	cpm/5 × 10^6 cells	224	10,487	9,583	2,840
5. 8-day BFU-E[27]	Bursts per 10^6 cells	0	205	150	62
6. 8-day ^{59}Fe[20]	cpm/10^6 cells	40	2,232	2,918	582
In Vivo Assays					
7. Polycythemic[13] mouse (^{59}Fe)	% Uptake	0.08	14.3	22.2	4.6
8. Appearance of nucleated red cells	Yes/no	No	Yes	Yes	Yes
9. Nude mouse tumors	Hematocrit	48	ND	68	57

In assays 1 through 4, standard Epo was added at a final concentration of 0.25 U/mL. HFTC-5 and TP-3 conditioned media (4-day harvest) were assayed at a final concentration of 10% vol/vol. In assays 5 and 6, the final concentration of standard Epo was 2.5 U/mL and conditioned media were concentrated tenfold (Amicon filtration) before use in the assay at 10% vol/vol. In assays 7 and 8, 0.25 units of standard Epo or 1 mL of conditioned media was injected subcutaneously into plethoric mice.[13] In assay 7, all samples were coded samples and were assayed "blind" by J.S. In assay 8, we tested whether inoculation of 1.0 mL of media into polycythemic mice[13] produced morphologically identifiable erythroblasts 48 hours later. In assay 9, we tested whether HFTC-5 and TP-3 were tumorigenic in nude mice and if so, whether tumor-bearing mice exhibited polycythemia. Cells (2 × 10^6) from each line were inoculated subcutaneously into three NIH nu/nu mice at 4 weeks of age. Controls were given a single injection of phosphate-buffered saline. Animals inoculated with HFTC-5 and TP-3 exhibited subcutaneous tumors eight weeks postinoculation. At this time, blood was collected from the tail and hematocrits (% packed cell volume) determined. The observed increase in hematocrits appeared to be due to increased red cell production, since no changes in the thickness of the white blood cell layer (buffy coat) were observed.

HFTC-5 media were unable to stimulate the growth of granulocyte-macrophage (GM) colonies, and therefore were presumably devoid of GM colony-stimulating factors.[26]

Neutralization of erythropoietic activity by anti-erythropoietin antiserum. Table 3 presents evidence that the erythropoietic activities in the media from HFTC-5 exhibited immunologic properties of Epo. Antiserum from rabbits that had been immunized with human urinary erythropoietin was previously shown to neutralize endogenous mouse Epo.[16,27] Conditioned media from HFTC-5 were mixed with this antiserum for a 30-minute incubation, after which goat antirabbit gamma globulin (GARGG) was added and the immune precipitate removed by centrifugation.[27] This approach was chosen instead of the alternative method of direct inoculation of the antiserum into the mouse without prior exposure to the hormone. Our rationale was that the erythropoietic agent in conditioned media might not be Epo, but only an enhancer or inducer of endogenous Epo. If such were the case, in vivo inoculation of antiserum would still abolish the erythropoietic activity. Because neutralization was accomplished in vitro before inoculation, we can conclude that the erythropoietic agent in the conditioned media is immunologically similar to Epo. That antigen–antibody interactions were responsible for the neutralization (rather than some other nonspecific inactivation by other agents in the antiserum) was indicated by our finding that pretreatment of the antiserum with GARGG abolished its Epo-neutralizing activity (Table 3). Three other immunology observations are relevant (data not shown). First, 0.3 to 0.8 U/mL of Epo has been detected in HFTC-5 conditioned media by radioimmunoassay. Second, the Epo antiserum recognized and neutralized erythropoietic activity recovered from the 43- to 45-kd area after gel filtration (see Fig 1) on Sephacryl S200. Finally, conditioned media from TP-3 and IW32 were also neutralized by this antisera. Collectively, these data indicate that the erythropoietic agent produced by these murine lines shares antigenic properties with human erythropoietin.

Physical properties of erythropoietic factors. To further assess the properties of these erythropoietic factors, conditioned media from HFTC-5 and TP-3 were tested after exposure to a variety of physical and enzymatic treatments (data not shown). The agent responsible for erythroid stimulation was stable to heat treatment (56 °C for one hour and boiling for five minutes), dialysis (molecular weight [mol wt] cutoff 30 kd), and more than five freeze-thaw cycles. Exposure of the conditioned media to degradative enzymes revealed that more than 90% of the in vivo[13] erythropoietic

Table 3. Neutralization of Erythropoietic Activity Produced by HFTC-5 by Erythropoietin Antiserum

Treatment	^{59}Fe Uptake in Polycythemic Mouse Assay (%)
Uninjected	0.08 ± 0.01
Normal rabbit serum + HFTC-5 media + GARGG	15.00 ± 1.47
Epo-antiserum + HFTC-5 media + GARGG	0.70 ± 0.28
(Epo-antiserum + GARGG), then + HFTC-5 media	9.40 ± 1.70

Rabbit erythropoietin antiserum[17] (0.2 mL) was added to 6 mL of test media and incubated at 37 °C for 30 minutes after which 0.5 mL of goat antirabbit gamma globulin (GARGG) was added. After further incubation at 37 °C for 30 minutes, precipitates were removed by centrifugation at 5,000 rpm for ten minutes. One milliliter of the supernatant was injected subcutaneously into plethoric mice. Two days later the mice received 1 µCi ^{59}Fe (ICN). Seventy-two hours subsequent to injection of the isotope, 0.5 mL of blood was removed and the percentage of ^{59}Fe uptake determined.[23] Standard error of the mean, six mice per assay.

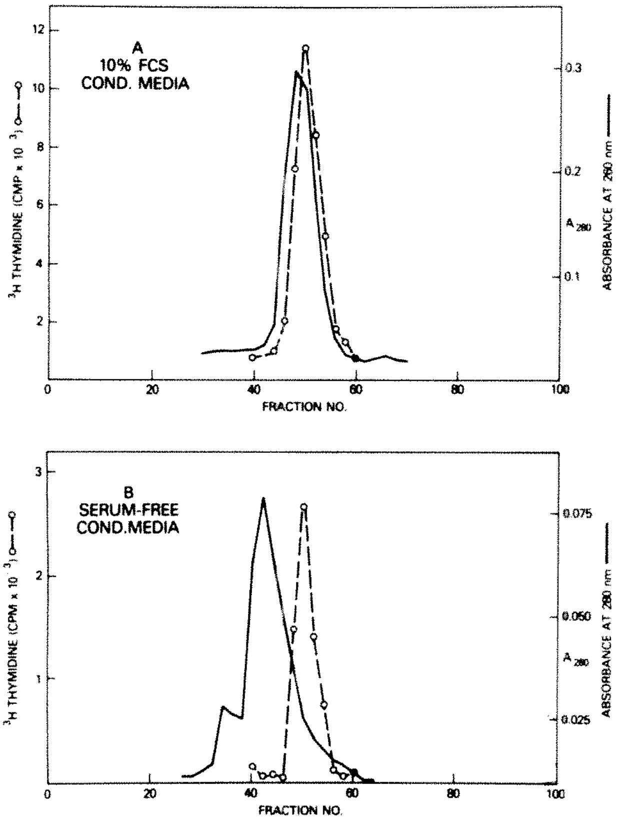

Fig 1. Gel filtration of HFTC-5 conditioned media. Conditioned media was prepared in the presence (A) or absence (B) of serum as described in Materials and Methods. Gel filtration was performed on Sephacryl S200 (79 × 2.5 cm). Fractions were tested for erythropoietic activity in the ^{3}H-tdr assay. When precalibrated at a flow rate of 1 drop per second, albumin (67,000 daltons) and ovalbumin (43,500 daltons) were recovered in fractions 43 and 53, respectively. The peak of erythropoietic activity was recovered between 43 and 45 kd in both (A) and (B).

activities were destroyed by trypsin and neuraminidase[17] but not by ribonuclease or deoxyribonuclease. On ammonium sulfate fractionation, more than 90% of the erythroid stimulating activity was precipitated at salt concentrations between 50% and 80% of saturation. Thus, in all cases, the murine erythroid factors exhibited physical properties indistinguishable from those reported by others for sheep and human Epo.[1]

Erythropoietic activity in TP-3 and HFTC-5 is not a virus. Because these cell lines were isolated from Friend virus-infected mice, an infectious virus might be the erythropoietic agent. However, erythropoietic activity was not removed from conditioned media of HFTC-5 and TP-3 by a centrifugation that pellets RNA tumor viruses (100,000 g for two hours[28]) (data not shown). Furthermore, the heat stability, mol wt estimates, enzyme sensitivity, and antibody neutralization studies are not consistent with the notion that the erythropoietic agents are RNA tumor viruses.

Production of erythropoietin by cloned erythroleukemia cell lines. Because HFTC-5 and TP-3 were independently derived from mice with Friend erythroleukemia, a high percentage of the cells resemble basophilic erythroblasts when stained with Wright's stain, and are lightly positive for spectrin,[6] it was considered likely that the cells making Epo were erythroid cells. However, because the cells were heterogeneous with respect to size and morphology, we have sought more rigorous evidence that the cells that secreted Epo were the same as cells with erythroid potential. A series of subclones was derived by two cycles of single-cell cloning and tested for Epo production and their potential to undergo terminal erythroid differentiation. Table 4 shows that every subclone of HFTC-5 and TP-3 produced Epo. In this experiment, the amount of Epo varied from 0.14 U/mL to 5.2 U/mL. It should be emphasized that the harvests in this experiment were random collections. Experiments are in progress to determine whether the level of production of a particular subclone is stable with passage. The table also shows that in the 20 subclones tested, all were positive for spectrin and were induced to differentiate by incubation in hemin.

Production and assay of Epo in serum-free cultures. Our attempts to grow HFTC-5 cells in serum-free culture have thus far been unsuccessful (C.E. has preliminary evidence that one clone of IW32 cells proliferated and produced Epo in serum-free media). In the present study, Epo continued to accumulate in cultures maintained for ten days in the absence of serum. Clone HFTC-5d (see Table 4) was seeded at 2 × 10^6 cells per line in Iscove's modified Dulbecco's medium supplemented only with 5 μg/mL trans-

Table 4. Production of Epo by Cloned Erythroid Cell Lines

	Epo (U/mL)	Benzidine Positive	Spectrin Positive
TP-3			
Subclone No.			
a	1.40	Yes	Yes
b	1.60	Yes	Yes
c	0.22	Yes	Yes
d	0.86	Yes	Yes
e	1.12	Yes	Yes
f	2.00	Yes	Yes
g	0.17	Yes	Yes
h	0.72	Yes	Yes
i	0.76	Yes	Yes
j	0.58	Yes	Yes
HFTC-5			
Subclone No.			
a	1.12	Yes	Yes
b	5.20	Yes	Yes
c	1.63	Yes	Yes
d	1.36	Yes	Yes
e	0.90	Yes	Yes
f	1.12	Yes	Yes
g	1.56	Yes	Yes
h	1.36	Yes	Yes
i	0.36	Yes	Yes
j	0.34	Yes	Yes

Random harvest of conditioned media was assayed in the tritiated thymidine uptake assay as described by Krystal.[14] More than 50% of the cells reacted with benzidine and with anti-spectrin antibody after four days' culture in medium containing 5 × 10^{-5} mol/L hemin.

ferrin and 5 μg/mL insulin. Assays of conditioned media collected on days 2, 4, and 10 gave Epo values of 0.22, 0.47, 0.86 U/mL in vitro and 0.10, 0.53, and 0.75 U/mL in vivo, respectively. Addition of 1% bovine serum albumin or increasing the level of transferrin or insulin did not lead to higher Epo levels.

When conditioned media were subjected to gel filtration, the in vitro erythropoietic activity was recovered between 43 and 45 kd whether the cells were cultured in the presence or absence of serum (Fig 1). The optical density profile, however, was quite different and indicated that gel filtration of serum-free material provided substantial purification of Epo from the bulk of the protein. These observations suggest that this murine Epo is secreted in a mature form and does not require modification or activation by serum factors.[29]

DISCUSSION

We previously reported[4] that an erythroleukemia cell line, identified by Dr Pierre Tambourin, released erythroid growth factors. In this report, we have attempted to extend our earlier observations by addressing four questions. (1) Is erythropoietin production a universal property of erythroid leukemia cell lines? (2) Is the erythropoietic factor erythropoietin? (3) Can erythropoietin be produced in serum-free cultures? (4) Are the factor-producing cells, indeed, erythroid?

We have now screened more than 70 murine erythroleukemia lines for production of erythroid growth factors. Four positive lines have been identified. HFTC-5, TP-3, and IW32 have consistently produced the highest quantity of Epo. It is interesting that all the positive lines were isolated from leukemic mice that had been infected with a replication-competent murine leukemia virus biologically cloned from Friend virus stocks. When inoculated into newborn NIH Swiss mice, F-MuLV induces a rapid erythroproliferative disease that culminates in splenomegaly, anemia, and death in 100% of the animals.[5] However, weekly transfusion of red blood cells when severe anemia develops leads to apparent amelioration of the symptoms and survival of the mice.[6] Three to 12 months after cessation of the transfusions, approximately half the mice again develop splenomegaly and anemia and, ultimately, die of leukemia. Intravenous inoculation of spleen cells from the relapsing mice revealed transplantable leukemic cells that produce fatal leukemia. From such transplantable leukemias, permanent erythroid lines have occasionally been established in culture, and as we report here, some but not all of these lines produce Epo. Thus far we have found three of eight lines to be positive, whereas our French collaborators have detected Epo production by two of five lines (P. Tambourin, personal communication, July 1985). Therefore, production of Epo at high levels (>0.1 U/mL) is not an invariant property of F-MuLV–induced erythroleukemia cells. Furthermore, none of the other virus-induced or chemically induced erythroleukemias or human erythroleukemia cell lines produced the hormone. Therefore, we can conclude that the production of Epo is not a universal property of erythroleukemia cell lines.

In the absence of the amino acid sequence of murine erythropoietin, we cannot unequivocally establish that this erythropoietic factor is a single molecular entity. Nevertheless, the erythroleukemia factor specifically stimulated erythropoiesis in nine distinct assays and was physically, biochemically, and immunologically indistinguishable from commercial sheep plasma Epo and human urinary Epo. Gel filtration, dialysis, and antibody neutralization data argue against the possibility that the erythropoietic activity is a prostaglandin, or cyclic adenosine monophosphate, or cyclic guanosine monophosphate, which have also been reported to influence erythropoiesis.[2] Therefore, we conclude that the erythropoietic molecule released by HFTC-5 and TP-3 is mouse Epo. Additional support for this conclusion comes from our purification studies (this issue) and studies by Choppin et al.[30]

Our correlation of Epo production with induction of benzidine-reactive material and spectrin in 20 cloned lines and our morphology studies all indicate that HFTC-5 and TP-3 cells are in fact erythroid precursors. We cannot, however, rule out the possibility that the clones were bipotential and could give rise to a small percentage of non-erythroid progeny.

The production of a red blood cell regulator by erythroid progenitors was an unexpected finding. Two explanations for this phenomenon may be suggested. First, since all the positive lines were derived from F-MuLV–infected mice, Epo production may be uniquely related to the pathology of F-MuLV disease. For example, insertion of all or part of F-MuLV or another virus may activate the erythropoietin gene or a regulatory gene that influences transcription of the Epo gene. Given the relatively high percentage of positive lines, such a finding would suggest the existence of preferred integration sites or "hot spots."

Second, it is possible that normal erythroid precursors may transiently secrete Epo at a certain stage of differentiation, and that HFTC-5, H-11, TP-3, and IW32 may represent erythroid progenitors "selected" at this stage. Although we have no information regarding the target cells for F-MuLV, earlier studies have indicated that the target cells for SFFV-induced erythroid transformation in vitro are confined to a relatively narrow window of erythroid differentiation. Thus, it is possible that F-MuLV provides a growth advantage (transforms) for erythroid precursors at a stage of differentiation in which transient autocrine production of Epo is part of normal development. According to this thesis, the Epo-negative lines would be presumed to have been transformed by F-MuLV at an earlier or later stage of differentiation. Lotem and Sachs,[31] on the basis of studies with transformed myeloid cells, suggested that the production of myeloid differentiative factors might be associated with normal myeloid differentiation. Others[32,33] have associated autocrine production of hematopoietic factors with the leukemic phenotype.

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A New Variant of Type II von Willebrand Disease With Aberrant Multimeric Structure of Plasma but Not Platelet von Willebrand Factor (Type IIF)

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A patient with a lifelong bleeding disorder was diagnosed as having Type II von Willebrand disease. The larger multimers of von Willebrand factor were absent from her plasma but present in platelets. A high-resolution electrophoretic technique was used to study the complex structure of individual von Willebrand factor multimers. In normal plasma, each multimer could be resolved into five bands: a more intense central one and four less intense, two moving faster and two slower than the central band. In normal platelets, each multimer could also be resolved into five bands. The central one had a mobility similar to that in plasma, whereas the four satellite bands had a mobility that differed from that of the corresponding plasma bands. In the patient, platelet von Willebrand factor antigen content and ristocetin cofactor activity were normal, and von Willebrand factor showed the same structure of individual multimers as seen in normal platelets. On the other hand, plasma von Willebrand factor antigen and ristocetin cofactor activity were decreased, and the structure of individual von Willebrand factor multimers was different from that of normal plasma and similar to that seen in normal and patient's platelets. After infusion of 1-deamino-8-D-arginine vasopressin, the largest von Willebrand factor multimers, as well as new satellite bands with a mobility similar to those in normal plasma, appeared in the patient plasma, and the levels of von Willebrand factor antigen and ristocetin cofactor activity became normal. Yet no relevant change in the prolonged bleeding time was observed. This new variant of von Willebrand disease, therefore, is characterized by the presence of a dysfunctional von Willebrand factor molecule that exhibits unique structural abnormalities in plasma but appears to be normal in platelets. The designation of Type IIF is proposed for this type of von Willebrand disease in accordance with the terminology that has been previously used.

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ANALYSIS OF THE MULTIMERIC STRUCTURE of von Willebrand factor (vWF) has proved essential for a more precise characterization of the molecular abnormalities of this glycoprotein in patients with von Willebrand disease.[1,2] Improvement in the electrophoretic resolution of the structural organization of individual vWF multimers[3,4] has recently been accompanied by the identification of new variant forms of von Willebrand disease.[4-9] It is apparent that the survey of large numbers of patients using refined analytical methods will lead to a better characterization of the congenital molecular defects of vWF and will help to explain the significance of the complex multimeric structure of this protein.

In this study we have used an improved high-resolution electrophoretic technique recently developed by some of us,[4] to define the structural vWF abnormality in a new variant form of Type II von Willebrand disease. The patient described here had a lifelong bleeding disorder and lacked the largest vWF multimers in plasma. These were present in platelets, however, and appeared in the circulation after administration of 1-deamino-8-D-arginine vasopressin (DDAVP). Moreover, the complex banding pattern of each multimer was distinctly aberrant in the patient's plasma but normal in her platelets. In keeping with terminology that has been previously used,[1,3-9] this new variant form has been designated Type IIF von Willebrand disease.

MATERIALS AND METHODS

Patient. The propositus was a 55-year-old Caucasian female who was referred to the Angelo Bianchi Bonomi Center in Milan, Italy, in March 1983 because of excessive bleeding during hysterectomy for fibromyoma. A lifelong history of easy bruising, epistaxis, and menorrhagia was reported. Two dental extractions had been accompanied by excessive bleeding five years before referral, but appendectomy and cholecystectomy were carried out without bleeding complications at ages 31 and 45, respectively. The mother, a brother, and a sister of the propositus did not report any bleeding tendency. The father died five years before our evaluation and had no history of bleeding. In May 1983, a surgical procedure was deemed necessary to correct a vaginal fistula diagnosed in the propositus. She was hospitalized and treated with DDAVP and cryoprecipitate to correct her bleeding tendency. DDAVP was also given subsequently on several occasions for experimental purposes. Informed consent was obtained from the patient and all normal controls before performing any experimental procedure, according to the Declaration of Helsinki.

Experimental procedures. The bleeding time was measured by performing two 1-cm long × 1-mm deep incisions in the forearm skin, using a template device (Simplate II, General Diagnostics, Morris Plains, NJ).[10] Blood was drawn from an antecubital vein, through a 19-gauge needle, into a polypropylene syringe and immediately transferred into polypropylene tubes containing 1/10th final volume of 0.129 mol/L trisodium citrate. The anticoagulant also contained protease inhibitors, namely, EDTA, *N*-ethylmaleimide, and leupeptin (Chemicon, El Segundo, Calif) to give final blood concentrations of 5 mmol/L, 6 mmol/L, and 1 mmol/L, respectively. Platelet-rich plasma was prepared by pooling the supernatants from three successive centrifugation steps, each performed at

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1,100 g for 45 seconds at room temperature (22 to 25 °C). Ristocetin-induced platelet aggregation (RIPA) was measured with graded amounts of ristocetin (Sigma Chemical Co, St Louis) as previously reported.[11] Platelet-poor plasma was prepared by centrifugation at 3,000 g for 20 minutes at 4 °C. It was then rapidly separated from the sedimented cells and either used for assay the same day or stored at −70 °C until tested. When necessary, frozen plasma was transported in dry ice. Factor VIII procoagulant activity (VIIIC) was assayed by a one-stage clotting technique[10]; vWF antigen by electroimmunoassay[10]; and the ristocetin cofactor activity of vWF, using formalin-fixed platelets.[12] Concentrations of these three activities were expressed in international units (IU) per deciliter, referred to pooled normal plasma calibrated against the First International Reference Preparation for Factor VIII-Related Activities in Plasma (National Institute for Biological Standards and Control, London). The activity of "average" normal plasma corresponds to 100 IU/dL. Platelets were counted in blood collected in EDTA, using phase contrast microscopy.[13]

Electrophoresis of vWF in agarose gels containing sodium dodecyl sulfate (SDS) was performed as previously described,[3] with the following modifications. Short gels were 22 cm wide × 9 cm long × 1.5 mm thick and were cast using either 1.2% high-gelling temperature agarose or 1.4% low-gelling temperature agarose (Sigma) on Gel Bond film (FMC Corp, Rockland, Me). The gels were allowed to polymerize at 4 °C for three hours before the pouring of the stacking gel, 2.5 cm long, consisting of 0.8% high-gelling temperature agarose (FMC). Ten sample wells, each 10 × 2 mm, were cut 1 cm from the stacking-resolving gel interface, and 20-μL samples were applied. Electrophoresis was performed horizontally, at a constant current of 0.41 mA/cm and constant temperature of 16 °C, in an LKB Multiphor apparatus (LKB, Pleasant Hill, Calif) until the tracking dye was 0.5 cm from the anodal wick (usually 18 hours). Long gels were 14 cm wide × 29 cm long × 1.5 mm thick and were cast using 2.2% low-gelling temperature agarose on Gel Bond kept between glass plates. The stacking gel was poured around an eight-well Teflon comb. Samples, 25 μL containing 30% glycerol, were applied into the wells. Vertical electrophoresis was performed in a Hoefer Scientific (San Francisco) SE 620 apparatus at 0.75 mA/cm across the gel for 18 hours or until the tracking dye was 1 cm from the bottom. After electrophoresis, the gels were fixed and reacted overnight with ^{125}I-labeled rabbit antihuman vWF antibody as previously described. Identification of vWF multimers using ^{125}I-labeled affinity-purified anti-vWF IgG was performed as described.[3]

The platelet content of vWF antigen and ristocetin cofactor activity was measured in platelets that had been washed free of plasma constituents and then lysed by a previously published procedure.[14] For analyzing the multimeric structure of platelet vWF, equal parts of platelet-rich plasma and a buffer solution containing 13 mmol/L sodium citrate, 28 mmol/L glucose, 123 mmol/L sodium chloride, 5 mmol/L disodium EDTA, 6 mmol/L *N*-ethylmaleimide, and 0.1 mmol/L leupeptin (pH 6.5) were mixed and then spun at 1,000 g for 25 minutes at 4 °C. After a second centrifugation, the pellet was resuspended in no more than 2 mL of a buffer composed of 10 mmol/L Trizma base, 140 mmol/L sodium chloride, 5 mmol/L disodium EDTA, 6 mmol/L *N*-ethylmaleimide, and 0.1 mmol/L leupeptin (pH 7.4, adjusted with HCl). Washed platelets were counted, and the suspension was adjusted to give a count of approximately 2×10^9/mL and then frozen at −70 °C and thawed five times. After the final thawing, the suspension was centrifuged at 10,000 g for five minutes at 4 °C. The supernatant was diluted in electrophoresis sample buffer containing SDS[3] and analyzed for multimeric composition. In some experiments, washed platelets were lysed directly in the SDS-containing buffer.

DDAVP was infused intravenously into the propositus at a dose of 0.4 μg/kg of body weight. Blood bank cryoprecipitate was administered at the dose of 20 to 40 IU of factor VIIIC activity per kg of body weight. Blood samples were obtained, and the bleeding time was measured before, during, and after the infusion, according to a published protocol.[15,16]

RESULTS

The relevant clinical and laboratory findings in the propositus and her family are summarized in Table 1. In the patient, low plasma levels of Factor VIIIC, vWF antigen, and ristocetin cofactor activity, as well as the prolonged bleeding time, were consistent with the diagnosis of von Willebrand disease. The mother, sister, and brother had normal results.

Multimeric analysis of vWF using large-pore agarose gels (cast with 1.2% high-gelling temperature agarose) showed absence of the largest, slower-moving forms in the patient's plasma but a normal pattern in platelets (Fig 1). All multimers present in normal plasma were also present in the patient's mother and brother (Fig 1), as well as in the sister (not shown). After using short gels of higher resolving power (cast with 1.4% low-gelling temperature agarose), it became apparent that the previously reported[3] "triplet" structure characteristic of individual vWF multimers in normal plasma was not seen in the patient (Fig 2). On the contrary, the typical pattern of normal platelet vWF, distinct from that

Table 1. Relevant Anamnestic and Laboratory Findings in the Propositus and Her Family

Family Member	Bleeding History	Bleeding Time (min)	Factor VIIIC (U/dL)	vWF Antigen (U/dL)	Ristocetin Cofactor	RIPA
Propositus	Positive	>30	45	36	15	Decreased
Mother	Negative	6	180	200	160	Normal
Sister	Negative	5	160	126	100	Normal
Brother	Negative	6	85	73	66	Normal
Normal subjects						
Mean		4.16	99	96	90	
Range		2.31–6.01	57–150	49–145	51–140	

RIPA (ristocetin-induced platelet agglutination) was measured by adding varying amounts of ristocetin to platelet-rich plasma and determining the concentration (expressed in mg/mL) that induced aggregation with an initial velocity corresponding to 30% of maximum (see ref 10). The results of VIIIC, vWF antigen, and ristocetin cofactor activity are expressed in international units (IU) per deciliter, where 100 units is the amount present in 1 dL of pooled normal plasma. Results in normal subjects represent either arithmetic mean ± SD (bleeding time, RIPA) or geometric mean with 95% confidence limits (all other measurements).

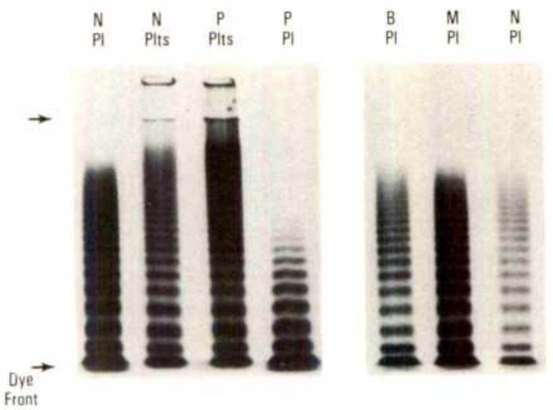

Fig 1. SDS gel electrophoresis in 1.2% high-gelling temperature agarose. The samples shown on the left are plasma (Pl) and platelets (Plts) from a normal individual (N) and the propositus (P); on the right, plasma from her mother (M) and brother (B). The arrow at the top indicates the interface between stacking and running gel; the one at the bottom indicates the dye front.

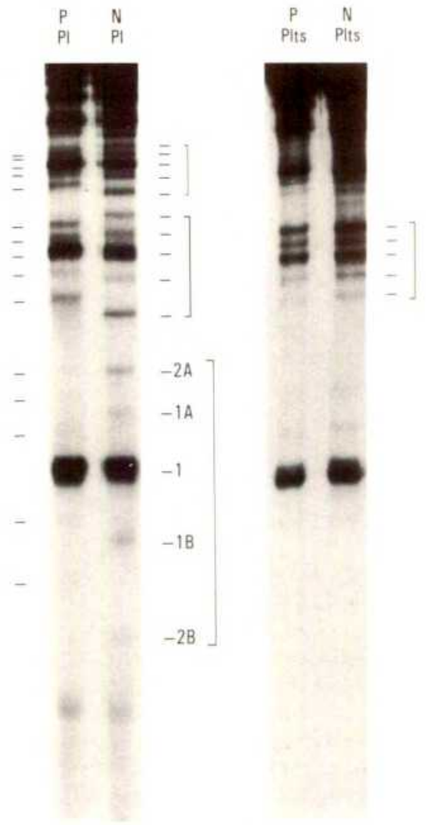

Fig 3. SDS agarose gel electrophoresis using a long gel of high resolving power. The samples shown are plasma (Pl) and platelets (Plts) from a normal individual (N) and the propositus (P). Marker bars denote the bands present in repeating multimers. The most intensely stained central band is indicated as 1; the two bands moving more slowly (above) are indicated as 1A and 2A; and the two moving faster (below) as 1B and 2B. The same bands can be detected in some multimers of increasing size but not in the larger multimers, which barely enter the running gel. Note the different mobility of satellite bands in the patient's plasma as compared with normal, but the similar mobility in normal and patient platelet vWF.

of plasma, was also seen in the patient's platelet vWF (Fig 2). The complex structure of individual multimers was further resolved by using longer agarose gels of high resolving power. In this system, the three to five smaller multimers appeared to consist of five bands, two migrating above and two below a most intense central band (Fig 3). The technique did not allow resolution of the complex structure of larger multimers, which barely entered this type of gel. In the plasma from more than 100 normal individuals, the mobility of satellite bands was consistently reproducible (data not shown; see also ref 4). When normal and patient plasma were compared side by side on the same gel, it became apparent that the central band of each vWF multimer had the same mobility in both cases, whereas the four satellite bands in the patient sample reproducibly migrated closer to the central band than did the normal counterparts (Fig 3). In this regard, the vWF structure in the patient's plasma was remarkably similar to that seen in platelet vWF. This finding was constantly observed on repeated analysis of two samples of the patient's plasma. On the contrary, the banding patterns of normal and patient platelet vWF were similar (Fig 3). The total protein content and vWF antigen levels were normal in the patient's platelets (0.1 IU of vWF antigen per milligram of platelet protein in the patient *v* 0.11 IU/mg in a normal control run at the same time; see also refs 14 and 16 for normal ranges). The ristocetin cofactor activity of the patient platelet vWF was also normal (0.12 IU/mg *v* 0.11 IU/mg). High-resolution gels demonstrated that the multimeric structure of plasma vWF in the patient's brother and mother was similar to normal (Fig 4). Therefore, the abnormality seen in the patient was not found in the other members of the family studied.

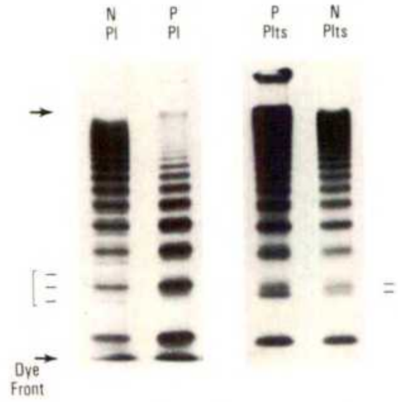

Fig 2. SDS gel electrophoresis in 1.4% low-gelling temperature agarose. The samples shown are plasma (Pl) and platelets (Plts) from a normal individual (N) and the propositus (P). The two arrows indicate, respectively, the interface between stacking and running gel (at the top) and the dye front (at the bottom). Marker bars denote the "triplet pattern" in normal plasma and the "doublet" in normal platelet samples.

The patient bled abnormally at surgery despite the administration of DDAVP, and satisfactory surgical hemostasis was achieved only after cryoprecipitate was given. No relevant shortening of the bleeding time was observed after

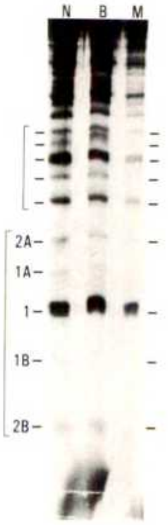

Fig 4. SDS agarose gel electrophoresis using a long gel of high resolving power. Plasma from the brother (B) and mother (M) of the propositus was analyzed along with a normal control plasma (N). The mobility of all bands appears to be similar in all cases. See Fig 3 for additional details on the interpretation of long gels.

DDAVP, even though vWF antigen and ristocetin cofactor activity were normalized (Table 2). Multimeric analysis of plasma vWF performed on short gels showed that the largest multimers appeared in the circulation immediately after the infusion of DDAVP and persisted for two to four hours (Fig 5). The complex structure of each multimer, however, remained abnormal. On long gels, additional bands previously not clearly detected became evident immediately after DDAVP infusion (Fig 6) and remained detectable for at least eight hours (not shown). Their mobility was similar to that of the satellite bands seen in normal plasma and contrasted with that of the abnormal bands still present in the post-DDAVP samples.

DISCUSSION

This patient, who had a lifelong bleeding tendency and a prolonged bleeding time, had low vWF antigen and ristocetin cofactor activity in plasma, contrasting with normal platelet levels of both moieties. In addition, there was a distinctive abnormality of plasma vWF multimeric structure, which

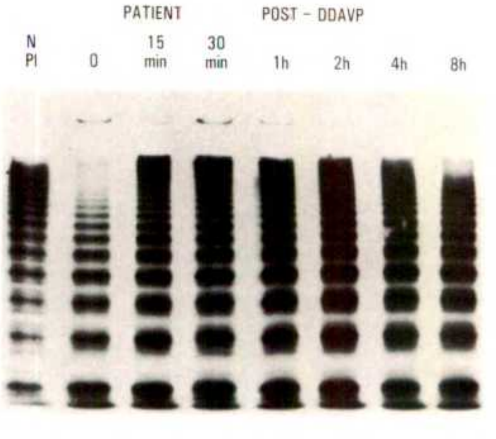

Fig 5. SDS agarose gel electrophoresis of samples obtained after DDAVP infusion. Normal plasma (NPl) is shown for comparison. Time measured in minutes (min) and hours (h) from the start of the administration (O) is reported. See Fig 2 for technical details.

became clearly evident using a combination of different agarose gels of varying resolving power.

Because of the absence of larger vWF multimers in plasma, this patient belongs to the category previously designated as Type II von Willebrand disease.[1] Five subtypes have been identified so far within this group (IIA, IIB, IIC, IID, and, recently, IIE), each of them characterized by distinct structural and functional abnormalities of vWF.[1,3,5-9,17] A discrepancy between the abnormal multimeric structure of vWF in plasma and the normal structure in platelets has been previously reported in two instances: Type IIB, as described by Ruggeri et al,[17] and Type IIA-3, reported by Weiss et al.[18] Type IIB patients are clearly different from this case because of the typical hyper-responsiveness of their platelet-rich plasma to low doses of ristocetin.[1,17] The opposite finding was observed in this patient, who had a decreased ristocetin-induced platelet agglutination. The cases called IIA-3 by Weiss et al also exhibited lack of larger vWF multimers in plasma, contrasting with a normal set of multimers in platelets. Because Weiss et al used low-resolution gels only and have not further characterized their variant, it cannot be established whether their patients are in some ways similar to the one described here.

The use of long agarose gels of high resolving power[4] has

Table 2. Relevant Laboratory Findings After DDAVP Infusion

Time	Factor VIIIC (U/dL)	vWF Antigen (U/dL)	Ristocetin Cofator (U/dL)	Bleeding Time (min)	Platelet Count ($\times 10^{-3}$/L)
0	45	36	15	>30	220
15′	87	40	47	—	182
30′	114	50	48	29	183
1 hr	107	56	55	—	198
2 hr	97	63	59	—	220
4 hr	85	73	68	>30	185
8 hr	59	72	44	—	196

DDAVP was infused at 0.4 μg/kg of body weight following the protocol described in ref 15. See Table 1 for normal values. Normal platelet count is 150 to 400 × 10^3/L.

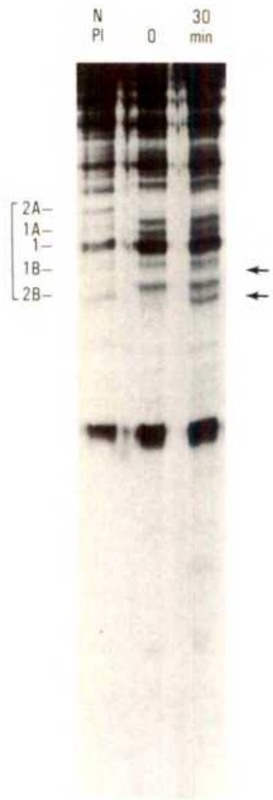

Fig 6. SDS agarose gel electrophoresis of samples obtained after DDAVP infusion. Refer to Fig 3 for details on the interpretation of long gels. Normal plasma (NPl) is shown for comparison. Plasma from the propositus was obtained before (O) and 30 minutes after DDAVP infusion. The marker bars denote the five bands present in the repeating vWF multimers. The two arrows on the right side indicate the post-DDAVP appearance in the patient's sample of two bands with mobility similar to normal bands 1B and 2B.

allowed a more precise definition of the complex banding pattern of individual vWF multimers in normal and patient's plasma and platelets. Five distinct bands could be resolved in each of the three to five smaller vWF multimers. The structure of the larger multimers could not be appreciated because they barely entered the small-pore long gels. Of the five bands, only the central, most intense one (sometimes resolved into a closely spaced doublet) showed similar mobility in both normal and patient's plasma vWF. The four satellite bands, on the contrary, had a distinctly altered mobility in the patient's plasma vWF as compared with normal. It is important to consider that the pattern seen in normal individuals was extremely reproducible, as demonstrated in well over 100 subjects. Therefore, the abnormal mobility of satellite bands seen in the plasma vWF of the patient reflects a unique, distinctive abnormality of this variant molecule. Also of interest is the fact that normal vWF of platelet origin exhibited a mobility of satellite bands different from that of the normal plasma counterparts but similar to that seen in the patient's plasma vWF. The patient's platelet vWF, in this regard, was identical to normal platelet vWF. At the present time, we cannot explain why plasma and platelet vWF show different mobility of the satellite bands seen in individual multimers, or why plasma vWF in the patient described here appears to have a structure similar to that seen in platelet vWF.

After infusion of DDAVP, additional satellite bands with a mobility similar to the satellite bands of normal plasma appeared transiently in patient's plasma, coexisting with the abnormal bands. Hence, vWF with an intact multimeric structure appears to be mobilized from storage sites by an appropriate stimulus such as DDAVP. At the present time, it remains unexplained why the multimeric structure of vWF becomes abnormal after release into the circulation. The abnormality could be detected despite addition to the plasma samples of a mixture of protease inhibitors that has been shown to partially prevent, in some cases, the absence of intermediate and larger vWF multimers in the plasma of patients with Type IIA von Willebrand disease.[19] At the present time, it is not possible to define the correlation, if any, between structural alteration of individual multimers and lack of the largest vWF multimers in plasma, despite their presence in platelets. In any case, our results demonstrate that the vWF molecule present in storage sites and mobilized by DDAVP was impaired in its function after release into the circulation. Larger vWF multimers, as well as satellite bands with a mobility identical to that of bands present in normal plasma, appeared in the patient's circulation and persisted for several hours after DDAVP infusion, but the bleeding time and the hemorrhagic tendency were not corrected, indicating that a dysfunctional protein was present.

The modality of genetic transmission of this newly described abnormality of vWF remains unclear. The mother and the two siblings of the propositus had a negative bleeding history and normal vWF. The father also had a negative bleeding history but had died before the patient came to our attention, and no data were available as to the structure of his vWF. The propositus had a lifelong bleeding tendency, so that the possibility of an acquired von Willebrand syndrome is remote. She had no descendants, however, to allow further insights into the mode of inheritance of this alteration. Altogether, the most likely possibility is that this new variant is transmitted as a phenotypically recessive trait.

In conclusion, these studies define a new variant form of von Willebrand disease, characterized by a distinctive aberration of vWF multimeric structure and impaired function of the molecule. In keeping with terminology that has been used previously, we propose that this variant be designated Type IIF von Willebrand disease.

ACKNOWLEDGMENT

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Activation of Plasminogen by Tissue Plasminogen Activator on Normal and Thrombasthenic Platelets: Effects on Surface Proteins and Platelet Aggregation

By Raphael B. Stricker, Dan Wong, Donny Tak Shiu, Patrick T. Reyes, and Marc A. Shuman

Tissue plasminogen activator (TPA) converts plasminogen to plasmin within the fibrin clot, thus localizing activation of fibrinolysis. To determine the extent to which platelets promote activation of plasminogen by TPA, we studied the interaction of TPA and plasminogen with unstimulated platelets. Normal washed platelets incubated in the presence of physiologic concentrations of plasminogen (180 μg/mL) and TPA (20 ng/mL) failed to generate plasmin activity. In contrast, incubation of platelets with TPA concentrations achieved during thrombolytic therapy (40 to 800 ng/mL) produced a tenfold to 50-fold increase in plasmin activity. After exposure to plasminogen and 200 ng/mL of TPA for one hour, platelets failed to agglutinate in the presence of ristocetin. Incubation of platelets suspended in autologous plasma with 400 ng/mL of TPA for one hour also inhibited ristocetin-induced agglutination. Exposure of platelets to plasminogen and increasing concentrations of TPA correlated with a decrease in glycoprotein Ib (GPIb) and an increase in glycocalicin, as shown by immunoblotting. The glycoprotein IIb/IIIa (GPIIb/IIIa) complex and a 250,000-dalton protein also disappeared from washed platelets after incubation with plasminogen and 200 ng/mL of TPA for one hour. These platelets failed to aggregate in the presence of adenosine diphosphate (ADP) or gamma thrombin, although aggregation in response to calcium ionophore A23187 and arachidonic acid remained intact. However, aggregation in response to all four agonists was normal when platelets were incubated with TPA in the presence of autologous plasma. Platelets from a patient with Glanzmann's thrombasthenia also generated plasmin in the presence of TPA. Hydrolysis of GPIb and inhibition of ristocetin-induced agglutination occurred to a lesser extent with these platelets than with control platelets. We conclude that platelets provide a surface for activation of plasminogen by pharmacologic amounts of TPA. Plasmin generation leads to degradation of GPIb and decreased ristocetin-induced agglutination in normal and thrombasthenic platelets, as well as degradation of GPIIb/IIIa in normal washed platelets and inhibition of ADP and gamma thrombin-induced aggregation. These findings suggest that pharmacologic concentrations of TPA may cause platelet dysfunction due to plasmin generation on the platelet surface.

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THROMBOLYSIS is a promising mode of treatment for acute myocardial infarction and thromboembolic disease.[1-5] Infusion of streptokinase, a nonspecific activator of fibrinolysis, is frequently associated with bleeding complications.[4,5] Tissue plasminogen activator (TPA) has recently been used in the treatment of acute myocardial infarction.[1] Activation of plasminogen by TPA requires fibrin. Thus, TPA provides a mechanism for specifically activating plasminogen at the site of thrombus formation. Localization of TPA to the fibrin clot is thought to reduce the risk of bleeding by preventing systemic activation of plasminogen and generalized fibrinolysis.[5]

Adelman et al[6] have reported that streptokinase interferes with ristocetin-induced platelet agglutination by means of plasmin-mediated degradation of platelet glycoprotein Ib (GPIb), the receptor for von Willebrand factor. Miles and Plow[7] recently showed that unstimulated platelets not only bind plasminogen, but also enhance its activation in the presence of a pharmacologic concentration of TPA, urokinase, or streptokinase. In the experiments described in this paper, we have examined the role of platelets in activation of plasminogen by TPA and the subsequent effect of plasmin on platelet membrane glycoproteins.

MATERIALS AND METHODS

Recombinant tissue-type plasminogen activator (500 μg/mL) was obtained from Genentech, Inc, Burlingame, Calif. Gamma thrombin (1.78 mg/mL), a generous gift of Dr John Fenton, New York State Department of Health, Albany, NY, was prepared as described.[8] Adenosine diphosphate ([ADP] Sigma Chemical Co, St Louis), arachidonic acid (Bio-Data, Willow Grove, Pa), calcium ionophore A23187 (Sigma), and ristocetin (Pacific Hemostasis, Bakersfield, Calif) were used for platelet aggregation studies. S-2251 was obtained from KABI via Helena Laboratories, Beaumont, Tex. Human Glu-plasminogen (1.25 mg/mL), kindly provided by Dr Craig Rice of Codon, Inc, South San Francisco, Calif, was prepared as previously described.[9] The plasminogen was stored in ε-aminocaproic acid (EACA) and dialyzed against Tyrode's buffer immediately before use to remove the EACA. Plasmin was purchased from Sigma and was used as a standard for activity, which was expressed in caseinolytic units (CU) per milliliter defined according to Squouris et al.[10] ^{14}C-Serotonin (5-hydroxytryptamine creatine sulfate-^{14}C) was obtained from Amersham Corp, Arlington Heights, Ill. Rabbit antibody against GPIb was the gift of Dr Joan Fox of the Gladstone Foundation Laboratories, San Francisco. Rabbit antibody against glycoproteins IIb and IIIa (GPIIb/IIIa)[11] was generously provided by Dr Rodger P. McEver of the University of Texas Health Science Center, San Antonio.

Control platelets and thrombasthenic platelets were prepared in the presence of prostaglandin E_1 (1 μg/mL) by isopycnic centrifugation over a Stractan gradient.[12] Thrombasthenic platelets were obtained from a patient with a bleeding time >30 minutes. These platelets failed to aggregate in the presence of ADP, collagen, or gamma thrombin, and they were shown to be more than 90% deficient in GPIIb/IIIa by immunoblotting with rabbit antibody raised against these glycoproteins.[11] The platelets were suspended in

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Tyrode's buffer at 200,000/μL and incubated with plasminogen and TPA for one hour at 37 °C as shown in Table 1.

After incubation, the platelets were centrifuged and the supernatants removed for assay of plasmin activity using substrate S-2251.[13] A standard curve was constructed using substrate and known amounts of plasmin. Platelets from (F) in Table 1 were resuspended in Tyrode's buffer, and aggregation using ristocetin (1.5 mg/mL), ADP (4 μmol/L), calcium ionophore A23187 (13 μg/mL), arachidonic acid (500 μg/mL), or gamma thrombin (2 μg/mL) was performed using a Payton dual-channel aggregometer (Payton Scientific, Inc, Buffalo, NY). Platelets from (A) and (B) in Table 1 were used for control aggregometry. In the case of platelet-rich plasma (PRP), control platelets were suspended in autologous plasma at 200,000/μL and incubated with TPA for one hour at 37 °C. Aggregation was then performed using the same agonist concentrations. PRP incubated with TPA buffer alone was used as a control.

Labeling of control platelets with ^{14}C-serotonin was performed as previously described.[14] After labeling in PRP, the platelets were isolated over Stractan and incubated with TPA and plasminogen for one hour at 37 °C as described. The platelets were centrifuged, and ^{14}C-serotonin release was measured in the supernatants. Radiolabeled platelets incubated with plasminogen or TPA alone were used as controls. The results were expressed as the percentage of ^{14}C-serotonin release above the percentage of release from controls.

Immunoblotting was performed as previously described.[15] Platelet samples from A, B, and D through H (Table 1) were solubilized in 2% sodium dodecylsulfate (SDS) at 1 × 10^9/mL. Fifty-microliter aliquots were electrophoresed in SDS-5% polyacrylamide gels under nonreducing conditions. After transfer to nitrocellulose, the platelets were incubated with rabbit anti-GPIb,[15] which recognizes platelet GPIb as well as glycocalicin on immunoblots, or with rabbit anti-GPIIb/IIIa.[11] The anti-GPIb antibody was used at a dilution of 1:500, while the anti-GPIIb/IIIa antibody was diluted 1:2,000 for immunoblotting. Antibody binding was detected using a biotin-avidin peroxidase system.[15]

RESULTS

Figure 1 demonstrates plasmin activity generated when platelets are incubated with a physiologic concentration of plasminogen[16] and increasing concentrations of TPA. Incubation of platelets with either plasminogen or TPA alone generated no plasmin activity. Similarly, plasmin activity was not seen after incubation of plasminogen with TPA in the absence of platelets or in the presence of washed red blood cells. Incubation of platelets and plasminogen with a physiologic concentration of TPA (20 ng/mL)[2] failed to generate protease activity. However, incubation with concentrations of TPA achieved during treatment of patients[2] produced increasing amounts of plasmin activity from 0.20 to 0.55 CU/mL. Platelets from four controls generated similar plasmin levels, and the results were reproducible in four experiments. Incubation of thrombasthenic platelets with plasminogen and TPA produced similar levels of plasmin activity from 0.10 to 0.45 CU/mL on three occasions. Plasminogen concentrations of 18 μg/mL yielded the same results as concentrations of 180 μg/mL in the presence of TPA, indicating a significant substrate excess under physiologic conditions. Incubation of labeled platelets with plasminogen and the highest TPA concentrations (400 and 800 ng/mL) resulted in the release of 20% to 30% of ^{14}C-serotonin, indicating partial platelet activation at these concentrations of TPA and plasminogen.

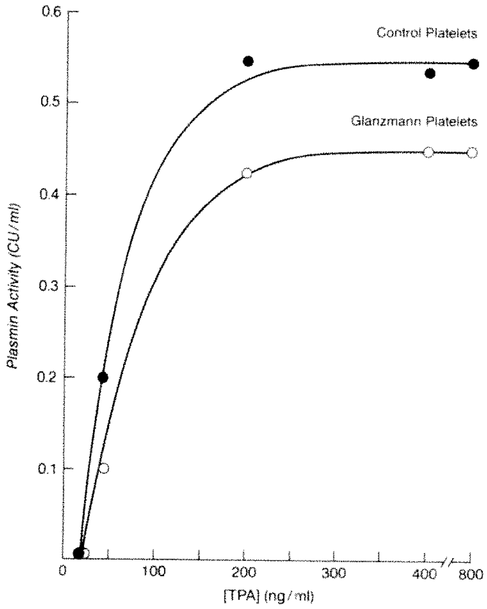

Fig 1. Plasminogen activation by TPA using control and thrombasthenic platelets. Washed platelets were incubated with plasminogen and increasing concentrations of TPA for one hour at 37 °C. The platelets were centrifuged and the supernatant was assayed for plasmin activity as described in Materials and Methods. Incubation of platelets with TPA alone, plasminogen alone, or plasminogen plus a physiologic TPA concentration (20 ng/mL) failed to generate plasmin activity. Platelet exposure to plasminogen and TPA concentrations of 40, 200, 400, and 800 ng/mL yielded plasmin activities of 0.20 to 0.55 CU/mL for control platelets and 0.10 to 0.45 CU/mL for thrombasthenic platelets.

Figure 2 shows the results of immunoblotting of platelet proteins after incubation of intact platelets with plasminogen and varying concentrations of TPA. In control platelets (Fig 2A), a major band of molecular weight (mol wt) 170,000 representing GPIb and a minor band of mol wt 250,000 are detected by the GPIb antibody. With increasing amounts of TPA, the 170,000- and 250,000-dalton bands decrease in intensity, and simultaneously, a 150,000-dalton band appears on the blots and then disappears at the highest TPA concentration. This suggests plasmin-induced degradation of

Table 1. Incubation Concentrations of Platelets, TPA, and Plasminogen

	A	B	C	D	E	F	G	H
Platelets/μL	200,000	200,000	—	200,000	200,000	200,000	200,000	200,000
TPA ng/mL	—	200	200	20	40	200	400	800
PLG μg/mL	180	—	180	180	180	180	180	180

PLG, plasminogen.

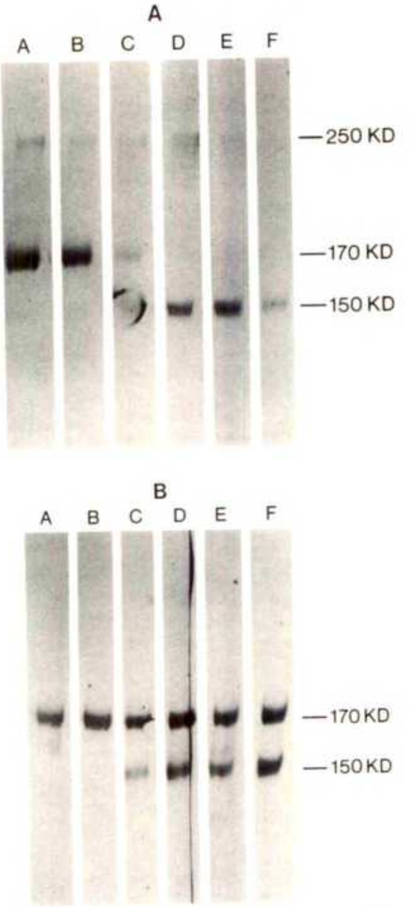

Fig 2. Effect of TPA and plasminogen on platelet GPIb. Washed platelets were incubated with plasminogen and increasing concentrations of TPA for one hour as described in Materials and Methods. The platelet proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with rabbit anti-GPIb antibody followed by biotin-conjugated goat F(ab)'$_2$ anti-rabbit IgG and avidin peroxidase. Lane A, platelets incubated with TPA 400 ng/mL alone; lane B, platelets incubated with plasminogen and TPA 20 ng/mL; lane C, platelets incubated with plasminogen and TPA 40 ng/mL; lane D, platelets incubated with plasminogen and TPA 200 ng/mL; lane E, platelets incubated with plasminogen and TPA 400 ng/mL; lane F, platelets incubated with plasminogen and TPA 800 ng/mL. (A) Normal platelets. (B) Thrombasthenic platelets.

GPIb and a higher mol wt protein with consequent generation of the proteolytic cleavage product glycocalicin.[6] Glycocalicin is also formed when thrombasthenic platelets are exposed to TPA and plasminogen, but the GPIb band is incompletely degraded (Fig 2B). Less GPIb is cleaved in the thrombasthenic platelets exposed to a plasmin concentration of 0.45 CU/mL (Fig 2B, lanes D through F) than in normal platelets exposed to a plasmin concentration of 0.20 CU/mL (Fig 2A, lane C). Thus, thrombasthenic platelets appear to be more resistant to the effect of plasmin than do control platelets. In addition, the 250,000-dalton protein is not seen in the thrombasthenic platelets. Thus, these platelets appear to be deficient in a higher-mol-wt protein that is degraded by plasmin.

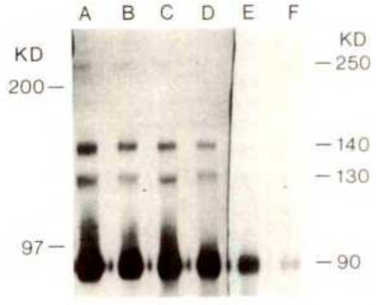

Fig 3. Effect of TPA and plasminogen on GPIIb/IIIa in normal platelets. The platelet preparation and conditions were as described in Fig 2. Lane A, platelets incubated with TPA 400 ng/mL alone; lane B, platelets incubated with plasminogen and TPA 20 ng/mL; lane C, platelets incubated with plasminogen and TPA 40 ng/mL; lane D, platelets incubated with plasminogen and TPA 200 ng/mL; lane E, platelets incubated with plasminogen and TPA 400 ng/mL; lane F, platelets incubated with plasminogen and TPA 800 ng/mL. Note bands at 250 kd, 140 kd, 130 kd, and 90 kd that disappear after exposure to TPA and plasminogen.

Figure 3 shows the results of immunoblotting using rabbit anti-GPIIb/IIIa. In control platelets exposed to TPA alone (lane A), four bands are detected by the GPIIb/IIIa antibody: the 250,000-dalton protein, two proteins of mol wt 140,000 and 130,000 corresponding to GPIIb,[11] and a 90,000-dalton protein corresponding to GPIIIa. After exposure of platelets to plasminogen and increasing concentrations of TPA, the four bands are no longer detectable (lane F). Thrombasthenic platelets do not contain these bands even without exposure to plasminogen and TPA (data not shown). Thus, exposure of platelets to TPA and plasminogen results in degradation of GPIIb/IIIa. In addition, the immunoblot findings with anti-GPIIb/IIIa antibody confirm the absence of the 250,000-dalton protein in thrombasthenic platelets, as suggested by the findings with anti-GPIb antibody.

Figure 4 shows the results of ristocetin-induced agglutination of washed platelets after exposure to plasminogen and 200 ng/mL of TPA for one hour. Ristocetin-induced platelet agglutination was inhibited by 80% compared with control platelets. In contrast, thrombasthenic platelets showed only a 30% inhibition of ristocetin-induced agglutination even after exposure to plasminogen and 400 ng/mL of TPA. However, the agglutination of untreated thrombasthenic platelets by ristocetin was 20% less than that of normal platelets in the washed platelet system. This difference is unexplained.

Figure 5 shows the effect of TPA on ristocetin-induced agglutination of normal platelets suspended in autologous plasma. Although 200 ng/mL of TPA had no effect on platelet agglutination, incubation of PRP with 400 ng/mL of TPA completely inhibited ristocetin-induced agglutination. These TPA concentrations generated plasmin activities of 0.04 and 0.20 CU/mL, respectively, in PRP. Thus, TPA exerts its effect both in a washed platelet system and in PRP, although in PRP, a higher concentration of TPA is needed to achieve the same effect as with washed platelets.

Figure 6 shows the aggregation curves of normal washed platelets in response to ADP, gamma thrombin, arachidonic acid, or calcium ionophore A23187. Exposure of platelets to

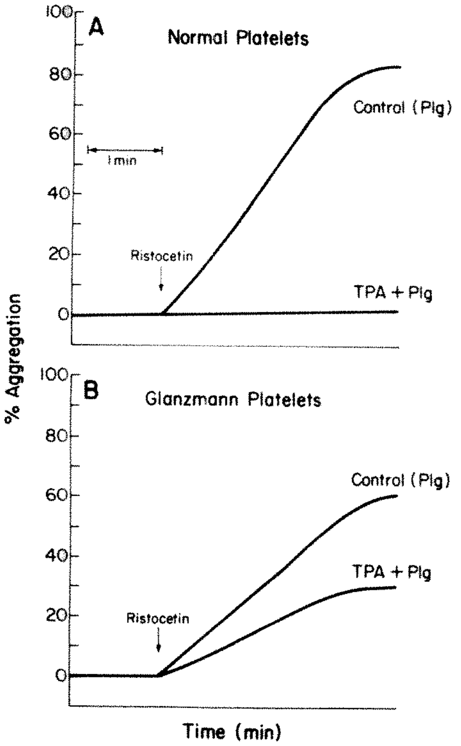

Fig 4. Ristocetin-induced agglutination of washed normal and thrombasthenic platelets after exposure to TPA. Washed platelets were incubated with plasminogen alone (control) or with plasminogen plus TPA 200 ng/mL (TPA + PLG) for one hour at 37 °C. After the platelets were washed, aggregometry was performed with ristocetin 1.5 mg/mL. Panel A, ristocetin-induced agglutination of normal platelets. Panel B, ristocetin-induced agglutination of thrombasthenic platelets.

plasminogen and 200 ng/mL of TPA for one hour at 37 °C inhibited the aggregation response to ADP and gamma thrombin by 80%. In contrast, platelet aggregation in response to arachidonic acid and calcium ionophore remained intact after TPA exposure. However, when PRP was substituted for washed platelets, aggregation in response to all four agonists remained intact, even after platelet exposure to 800 ng/mL of TPA (data not shown).

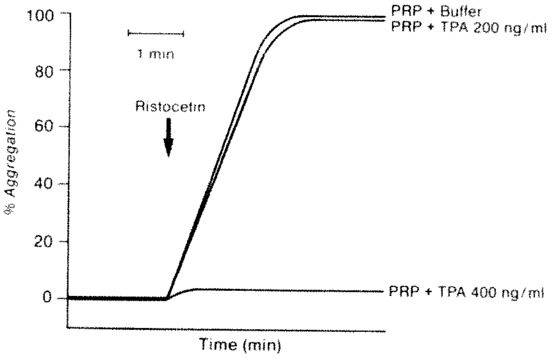

Fig 5. Ristocetin-induced agglutination of PRP. Platelets were suspended in autologous plasma at 200,000/μL and incubated with TPA 200 or 400 ng/mL for one hour at 37 °C. Ristocetin 1.5 mg/mL was then added, and aggregometry was performed. PRP incubated with TPA buffer alone was used as a control.

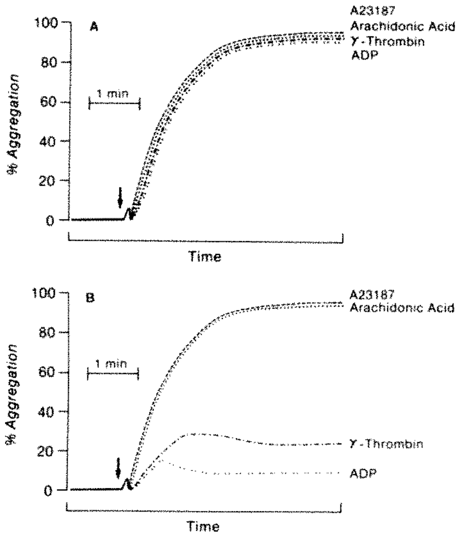

Fig 6. Washed platelet aggregation in response to ADP, gamma thrombin, arachidonic acid, and calcium ionophore A23187. Washed platelets were incubated with TPA alone (control) or with plasminogen plus TPA 200 ng/mL for one hour at 37 °C. Panel A, control aggregation curves. Panel B, platelet aggregation after exposure to plasminogen and TPA.

DISCUSSION

In this study, we have shown that washed platelets incubated in the presence of plasminogen and pharmacologic concentrations of TPA produced a tenfold to 50-fold increase in plasmin activity. Exposure of platelets to TPA and plasminogen resulted in a decrease in GPIb and an increase in glycocalicin, as shown by immunoblotting. The platelets failed to agglutinate in the presence of ristocetin after exposure to pharmacologic concentrations of TPA. In addition, GPIIb/IIIa was no longer immunologically detectable in these platelets, and the aggregation response to ADP and gamma thrombin was inhibited. Thrombasthenic platelets generated slightly reduced amounts of plasmin in the presence of TPA, and ristocetin-induced agglutination was not inhibited to the same extent as in normal platelets. By immunoblotting, the thrombasthenic platelets also appeared to be less susceptible to degradation of GPIb.

Our findings have three significant implications. First, we have shown that unstimulated platelets incubated with a physiologic concentration of TPA do not generate plasmin. Therefore, under physiologic conditions, platelets do not provide a mechanism for plasminogen activation by TPA. Second, it appears that unstimulated platelets can interact with pharmacologic doses of TPA with consequent plasmin generation. Third, the amount of plasmin generated by low therapeutic levels of TPA is capable of degrading GPIb, the von Willebrand factor binding site on platelets, thereby inhibiting platelet adhesion. GPIIb/IIIa also appears to be degraded by plasmin, although this does not occur in PRP.

Thus, TPA has the potential to cause platelet dysfunction at concentrations well within the therapeutic range[2] by means of plasmin generation and degradation of GPIb.

Our study suggests that platelet-dependent activation of plasminogen by TPA does not require GPIIb/IIIa. However, thrombasthenic platelets appear to be less susceptible to the action of plasmin on GPIb, since there was less inhibition of ristocetin-induced agglutination and less degradation of GPIb in these platelets at a moderate plasmin concentration (0.45 CU/mL). These findings imply that GPIIb/IIIa may be involved in the interaction of plasmin with platelets. Alternatively, thrombasthenic platelets may contain more GPIb than do control platelets, so that cleavage of this membrane glycoprotein by plasmin is incomplete. The residual GPIb would therefore be sufficient to allow platelet agglutination by ristocetin even in the presence of moderate levels of plasmin activity.

An additional finding in normal platelets is that a mol wt = 250,000 protein disappears in the presence of plasmin. In contrast, the 250,000-dalton band is not seen in thrombasthenic platelets even before exposure to plasmin. Furthermore, the 250,000-dalton protein was not detected by immunoblotting in platelets from four other thrombasthenic patients (data not shown). The nature of this protein and its role in platelet function are unknown. It represents either an additional protein that is decreased or missing in thrombasthenic platelets or a dimer of GP IIb-IIIa.

Previous studies using rabbit platelets[17] or human platelets[18] have shown that plasmin levels of 0.9 CU/mL or more can directly cause platelet activation and aggregation. Our results demonstrate that TPA does not generate enough plasmin to cause direct platelet aggregation, in accord with the observation of Schafer and Adelman[19] that plasmin concentrations of 0.5 CU/mL do not aggregate washed human platelets. However, at this plasmin concentration, partial platelet activation does seem to occur, as evidenced by ^{14}C-serotonin release. Our findings differ from those of Deguchi et al,[20] who described the interaction of a platelet extract with *Lys*-plasminogen in the presence of trace amounts of TPA. In contrast, our study used intact platelets with physiologic amounts of Glu-plasminogen and pharmacologic concentrations of TPA. Thus, plasmin generation in moderate quantities appears to occur on the platelet surface in the presence of TPA.

A major question concerns the applicability of our findings to in vivo platelet function in the presence of TPA. We know of no previous study in which platelet function was examined during TPA administration. Adelman et al[6] showed that when platelets are incubated with streptokinase at concentrations producing as little as 0.05 CU/mL of plasmin, ristocetin-induced platelet agglutination is inhibited. Little data exists on in vivo correlates of plasmin activity, however.[19] Our results using PRP suggest that plasmin generated by TPA levels achieved during thrombolytic therapy may interfere with platelet adhesion despite the presence of circulating plasmin inhibitors. However, we could show no effect on platelet aggregation in response to ADP, gamma thrombin, arachidonic acid, or calcium ionophore when PRP was substituted for washed platelets. Further studies in patients receiving TPA therapy are necessary to determine whether TPA interferes with platelet function by means of plasmin generation in vivo as well as in vitro.

In summary, we have shown that TPA interaction with plasminogen and washed platelets leads to plasmin generation, inhibition of platelet aggregation, and degradation of glycoproteins Ib and IIb/IIIa. Plasmin is generated on control and thrombasthenic platelets, but the effect of plasmin on thrombasthenic platelets appears to be decreased. The mechanism of TPA interaction with platelets and the risk of bleeding due to this interaction remain to be determined.

ACKNOWLEDGMENT

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Human T Lymphotropic Virus Type III Infection of Human Alveolar Macrophages

By Syed Z. Salahuddin, Richard M. Rose, Jerome E. Groopman, Phillip D. Markham, and Robert C. Gallo

The human T-cell lymphotropic virus type III (HTLV-III) is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) and preferentially infects T4 lymphocytes. Other cell types, notably B lymphocytes and other nonlymphoid cells, also have been reported to be infected in vitro by HTLV-III. We now report on the susceptibility of human pulmonary macrophages to infection with HTLV-III in vitro. Alveolar macrophages infected with HTLV-III produced low levels of virus that could be transferred to allogeneic human peripheral blood mononuclear leukocytes as long as 2 weeks after initiation of infection. Unlike HTLV-III infection of T lymphocytes, macrophages appeared more resistant to viral-mediated cytopathic effects. Primary cultures of pulmonary macrophages from two of four patients with AIDS spontaneously produced low levels of virus detected as precipitable reverse transcriptase activity, suggesting that these cells were infected in vivo. Because tissue macrophages are long-lived cells, they may act as a reservoir of HTLV-III, capable of transmitting the virus to other susceptible cells such as T lymphocytes, causing periodic low-level viremia. Macrophage infection with HTLV-III may be one mechanism for the establishment of viral persistence in infected hosts.

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THE human T lymphotropic virus type III (HTLV-III) has been demonstrated to be the primary causative agent of acquired immunodeficiency syndrome (AIDS) and related disorders.[1-4] HTLV-III has been recovered from several body tissues and fluids, including peripheral blood lymphocytes, cell-free plasma, lymphocytes in saliva, cerebrospinal fluid (CSF), tears, and semen.[4-5] Preliminary studies suggest that the receptor for HTLV-III infection is related to or closely associated with the surface antigen T4 found on helper/suppressor T cells.[6-8] In vitro, B lymphoblastoid cell lines, and Epstein-Barr virus (EBV)-transformed fresh lymphocyte have been infected with HTLV-III,[9,10] and a recent report suggested the possible infection of human macrophages.[11] Pathologic studies of simian brains of animals infected with simian T lymphotropic virus type III (STLV-III), a retrovirus related to HTLV-III, show by electron microscopy viral particles in macrophages.[12] Furthermore, nonlymphoid cells in the brain, possibly glial cells, are infected with HTLV-III in some patients with AIDS and dementia.[13] These studies suggest that a number of cells other than T cells may be infected with HTLV-III. We now report on the infection of human alveolar macrophages in vitro and provide suggestive data that these cells may be infected in vivo as well.

MATERIALS AND METHODS

Pulmonary macrophages were harvested from healthy volunteers and from patients with AIDS by broncholavage using previously described methods.[14] Informed consent was obtained from all participants and the procedures were approved by the Institutional Review Board of the New England Deaconess Hospital. The adherent cell population was obtained from the lung by incubation on plastic and multiple washes with phosphate-buffered saline (PBS) were performed to dislodge any nonadherent cells. The resulting adherent population was studied morphologically with inverted and phase contrast microscopy and tested for phagocytic capacity for 1.1-μm latex beads.[15,16] The adherent cells were tested for nonspecific esterase (naphthol acetate esterase).[17] Pulmonary macrophages were also assayed for the expression of cell surface antigens OKT1, OKT3, OKT4, OKT8, and OKT11 (Ortho Pharmaceuticals, Raritan, NJ); Leu 1, Leu 3a, Leu4, and Leu 12 (Becton Dickinson, Mountain View, Calif); and B1 and B4 (Coulter Immunology, Fla) as previously described.[18] To minimize the possible nonspecific staining due to the use of murine monoclonal antibodies, antimouse immunoglobulin conjugate was included in immunoassays. Adherent cells from healthy volunteers as well as AIDS patients were infected at days 3 through 5 after plating. These cells were incubated at 37 °C in RPMI 1640 medium after diethylaminoethanol (DEAE) treatment for 20 minutes and then cultured with standard titrated infectious alliquots of HTLV-III harvested from H9 cells.[3] Controls consisted of DEAE-treated macrophages exposed to the supernatants obtained from uninfected H9 cells. Productive infection with HTLV-III was measured by quantitation of reverse transcriptase activity in supernatant fluid[2-5] under the assay conditions used; a cell culture was judged positive when the cpm using $dT_{15} \cdot rA_n$ primer template was $\geq 5{,}000$ and was greater than or equal to threefold higher than cpm using $dT_{15} \cdot dA_n$ as primer template. Staining of target pulmonary macrophages with monoclonal antibodies to the HTLV-III correlated with p24 and p15 antigens to determine the percentages of positive cells.[2-5] Electronmicroscopy and transmission of HTLV-III from supernatant fluids of human alveolar macrophage cultures to normal peripheral blood lymphocytes was performed as previously described.[2-5]

Characterization of Adherent Cells

Morphology. Standard differential counts were performed on Wright's-Giemsa–stained cytocentrifuge-prepared cells to determine the gross morphology of the cell population and their state of differentiation. Myeloid cells through the metamyelocyte stage were classified as immature.

Cytochemistry. Published procedures were followed for the cytochemical evaluation of fresh and cultured cells. The tests performed included stains for naphthyl ASD chloracetate esterase,[14] α-naphthyl acetate esterase,[14] acid phosphatase,[15] and myeloperoxidase.[16]

Nitroblue tetrazolium reduction. The nitroblue tetrazolium (NBT) reduction test was performed on suspension and adherent

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cells with or without the addition of freshly diluted tetradecamylphorbal acetate (TPA), as previously described.[13,17] Cells were incubated for 25 minutes at 37 °C in media containing 0.2% NBT and the desired concentration of TPA. Cells were rinsed twice with media, pelleted on microscope slides using Shandon-Elliot cytospin, and stained with Wright's-Giemsa. Cells with dark formazan deposits were scored positive.

Phagocytosis. Suspension cells were mixed with either sterile carbonyl iron (Technicon Instruments Corp, Tarrytown, NY) at a concentration of 10% (vol/vol) or with formalin-fixed *Candida albicans* at a concentration of 4 × 10^6/mL with or without the addition of freshly diluted TPA, and incubated at 37 °C for 25 minutes. After incubation, cells were rinsed twice with media, and cytocentrifuge smears were prepared; these were stained with Wright's-Giemsa before evaluation. Adherent cells on the plastic surface of culture flasks were treated with either carbonyl iron or *C albicans*, rinsed, and evaluated directly under an inverted microscope after Wright's-Giemsa staining.

Detection of EBV antigens and antibodies. Serum from donors were tested for the presence of antibodies reactive against Epstein-Barr virus nuclear antigen (EBNA) and EBV-virus capsid antigen (VCA), and cells were tested directly for these antigens by published procedures.[19]

Rosette formation. Leukocytes were tested for their ability to form rosettes with either sheep or bovine erythrocytes either directly (E rosette) after treatment of erythrocytes with anti-sheep or bovine erythrocyte serum (EA rosettes) or addition of C_5-deficient mouse complement to the EA preparation (EAC rosettes) by published procedures.[20]

Surface-bound immunoglobulin. The presence of cell surface-bound Ig (SIg) was determined by direct procedures using fluorescence-labeled $F(ab')_2$ fragments of anti-human IgG, A, and M.

Monoclonal antibody profile. Murine monoclonal antisera reactive with peripheral blood monocytes[22] was used to detect monocyte-macrophages. Reacting cells were identified using FITC-labeled anti-murine IgG, $F(ab')_2$-specific serum.

RESULTS

The adherent cell cultures obtained by broncholavage were tested following three to five days in cell cultures. At this time they were >98% monocytic, (>94% phagocytic for latex beads, and >99% positive for nonspecific esterase, indicating a nearly uniform population of alveolar macrophages). Furthermore, these cells were negative for T and B cell-related antigens OKT1, OKT3, OKT8, and OKT11; Leu 1, Leu 3a, Leu 4, and Leu 12; and B1 and B4. A low percentage of these adherent cells (2% to 3%) expressed the T4 antigen. This did not appear to be the result of low-level contamination of macrophage cultures with lymphocytes since these studies were performed on rigorously prepared and characterized adherent cells after 3 to 5 days in culture. One hundred percent of these adherent cells were positive for OKM1, M5 (Ortho Diagnostic), Leu M1, and M3 (Beckton Dickinson), and MO1 and MO3 (Coulter Immunology) monoclonal antibodies directed against monocytoid/macrophage cells. This further indicates lack of contamination of the cultures with lymphocytes.

The results of infecting pulmonary macrophages from normal donors and AIDS patients in vitro are shown in Table 1 and Fig 1. Primary macrophage cultures from AIDS patients demonstrated low levels of reverse transcriptase activity (threefold to fivefold background) observed in two of four cases, suggesting the presence of cellular infection in vivo. Despite this suggestion of viral infection, HTLV-III (or other viruses) could not be visualized by electronmicroscopy, and attempts to transmit HTLV-III from these cells to normal T cells were unsuccessful.

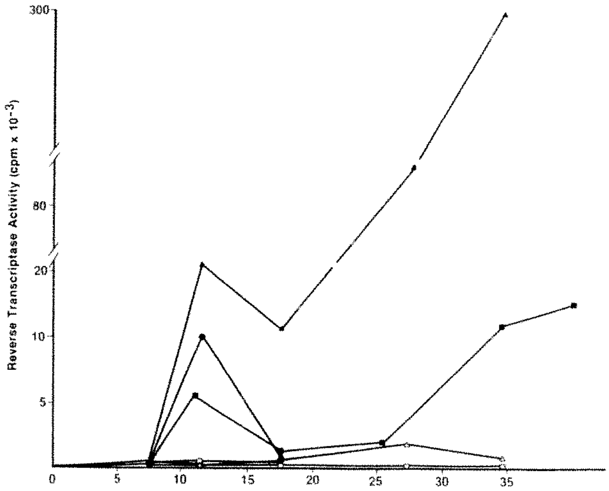

Fig 1. **Human alveloar macrophages obtained by broncholavage from normal volunteers were placed in tissue culture in RPMI 1640, and the adherent macrophages were infected with HTLV-III. Several of these specimens were tested as shown here for release of reverse transcriptase activity (RT). These cells were infected on day 5 and tested for RT at various time intervals for virus release. On day 22, fresh mononuclear peripheral blood leukocytes were added to these infected macrophages with a consequent rise in virus release. Uninfected cells from donor 5 □—□; cells infected with HTLV-III from donor 5 ■—■; uninfected cells from donor 6 △—△; cells infected with HTLV-III from donor 6 ▲—▲; uninfected cells from donor 7 ○—○; cells infected with HTLV-III from donor 7 ●—●.**

Pumonary macrophages from both AIDS patients and normal donors were susceptible to HTLV-III infection in vitro. As shown in Fig 1, 3 to 5 days after introduction to cell culture, adherent pulmonary macrophages from normal donors were exposed to HTLV-III, and levels of reverse transcriptase activity were detectable within 7 days. This production of virus was then amplified by addition to the culture of lectin-stimulated peripheral blood mononuclear cells from allogeneic donors on day 22 of culture. A marked increase in HTLV-III production was then obtained as detected by both release of precipitable reverse transcriptase activity and increase in the percentage of suspension cells expressing HTLV-III core-related proteins p15 and p24 (Table 1). The adherent cell population did not demonstrate gross cytopathic changes morphologically postinfection and was viable by trypan-blue exclusion for >65 days. Following HTLV-III infection of the adherent macrophage population, ~18% to 30% of the cells expressed HTLV-III core-related antigens, a much higher proportion of the macrophages than the T4 antigen-expressing population (2% to 4%).

DISCUSSION

These studies demonstrate that human pulmonary macrophages obtained from both normal volunteers and patients with AIDS are permissive for infection with HTLV-III.

Table 1. Infection of Alveolar Macrophages From Normal Donors and AIDS Patients HTLV-III, on Day 12 Postinfection

		Uninfected Cells		HTLV-III Infected Cells		
					IFA (% Positive)	
Specimen[*] Source	Patient No.	RT[†] Activity (cpm × 10^{-3})	IFA[‡] OKT4 (% Positive)	RT Activity (cpm × 10^{-3})	p15	p24
AIDS	1	3	<2	6	ND	ND
	2	–	ND	+	13	21
	3	±	<4	+	18	19
	4	–	ND	+	ND	ND
Healthy heterosexual donors	5	–	<4	6	30	29
	6	–	<3	11	25	18
	7	–	<4	23	19	24
	8	–	<4	5	29	32

ND, not done.

[*] Adherent cell cultures were established as described. These cells were >97% positive for nonspecific esterase (naphthyl acetate esterase) and ability to phagocytize latex beads; they were negative for T cell (eg, OKT1, or T3, OKT4, OKT8, Leu 1, Leu 3, and Leu 4 and for B cell (Leu 12, B1, and B4) antigens.

[†] Reverse transcriptase activity released into supernatant fluids of cell culture was determined as previously described: + positive, – negative, ± low levels of activity. These determinations were made every 5 days.

[‡] HTLV-III specific protein (p15, p24), T cell, and B cell markers were determined by immunofluorescence assays (IFA) as described.

Therefore, like retroviruses of other mammalian species, particularly visna and STLV-III, HTLV-III is capable of productively infecting mononuclear phagocytes. This infection appears to differ from that of lymphocytic cells in that the viability of the infected macrophages in vitro does not appear to be as adversely affected by HTLV-III. It is reasonable to speculate that mononuclear phagocytes may be infected in vivo in patients with HTLV-III–related diseases (HRD). The data of Shaw and colleagues demonstrating HTLV-III proviral DNA in nonlymphoid cells in brain tissues obtained from patients with AIDS-related dementia further support the concept that there are nonlymphoid targets of this retrovirus.[13] Because macrophages are relatively long-lived cells capable of close interaction with lymphocytes, and do not appear in vitro to be as susceptible to HTLV-III–induced cytopathic effects as do lymphocytes, it is possible that infected macrophages in vivo could propagate viral infection in the hosts by transfer of virus to lymphocytes. HTLV-III infection of mononuclear phagocytes could thereby be one mechanism for the establishment of viral persistence.

It is still undetermined whether in vitro HTLV-III enters the macrophages by specific interaction with a cell surface receptor, possibly related or unrelated to the T4 antigen, or whether the virus is simply phagocytosed and maintained in a viable state within vacuoles without integration as proviral DNA. Studies are underway to address these questions.

Because macrophages play an important role in the defense against certain neoplastic and infectious processes, alterations in these activities could contribute to host immunosuppression and further predispose to the development of malignancy and opportunistic infections. This is particularly clinically evident with respect to pulmonary macrophages in that patients with AIDS often manifest opportunistic infections of the lung. In this regard, it has been demonstrated that certain monocyte-macrophage functions such as antigen presentation are deficient in cells obtained from individuals with AIDS and related disorders.[19]

Strategies for anti-HTLV-III viral therapy, as well as immunomodulatory therapy, in patients with AIDS requires full definition of the target cells of this virus and possible reservoirs in vivo of viral infection. Our observations that in vitro pulmonary macrophage can be infected with HTLV-III and appear to be less susceptible to the cytopathic effects of this retrovirus suggest that tissue macrophages should be considered as potential reservoirs of HTLV-III in vivo.

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Cytochemical, Immunologic, Chromosomal, and Molecular Genetic Analysis of a Novel Cell Line Derived From Hodgkin's Disease

By Hiroshi Kamesaki, Shiroh Fukuhara, Eiji Tatsumi, Haruto Uchino, Hirohiko Yamabe, Hiroshi Miwa,
Shigeru Shirakawa, Masakazu Hatanaka, and Tasuku Honjo

A novel cell line, KM-H2, was established from the pleural effusion of a patient with Hodgkin's disease of mixed cellular type. Multiple phenotypic studies were carried out with this cell line. Acid phosphatase and nonspecific esterase activities were detected. Rosette formation with T lymphocytes and the receptors for C3b and Fc portion of IgG were positive. Among the antigens tested with a total of 22 monoclonal antibodies defining hematopoietic cell subsets or lineages, Ki-1, Leu-M1, MCS1, HLA-DR, and OKT9 antigens were found to be positive. The other antigens reportedly specific for T cells, B cells, natural killer (NK) cells, monocytes, interdigitating reticulum (IR) cells and dendritic reticulum cells were negative. These phenotypic features were identical to those of the Sternberg-Reed (SR) and Hodgkin (H) cells in the fresh materials reported by other researchers. Moreover, the KM-H2 cells and the parental pleural effusion cells shared several structural chromosome anomalies. These findings indicated that the KM-H2 cells are derived from the SR and H cells. Molecular genetic analysis of the KM-H2 cells disclosed that the human immunoglobulin J_H gene was rearranged but not the JK gene, and that the human T cell receptor β chain gene was of the germline type. Based on these properties of the KM-H2 cells, Hodgkin's disease may be derived from a cell lineage other than T cell or B cell.

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ALTHOUGH the Sternberg-Reed (SR) and Hodgkin (H) cells are regarded as the tumor cells in Hodgkin's disease, their nature and origin remain unclear. To clarify this question, a number of ultrastructural, histochemical, and immunologic studies have been performed on histological sections or after separation from biopsy materials. As a result, several cell lineages of the hematopoietic system have been suggested as candidates for the origin of the SR and H cells: T lymphocytes,[1] B lymphocytes,[2,3] macrophages,[4,5] granulocytes,[6] and dendritic reticulum cells,[7] as well as interdigitating reticulum (IR) cells.[8]

Faced with the difficulties of characterizing the SR and H cells, many investigators have attempted to establish in vitro cell lines derived from the SR and H cells. However, this has been hampered because these cells usually constitute only a minor population and seem to grow poorly in the hitherto available culture systems. Several permanent cell lines have been reported, but most of these do not appear to have arisen from typical SR and H cells, with the exception of the L428 cell line.[9]

We report here a novel cell line, KM-H2, established from the pleural effusion of a patient who was initially diagnosed as having Hodgkin's disease of mixed cellular type. We also describe its cytochemical, immunologic, and molecular genetic properties, and discuss the cellular origin of Hodgkin's disease.

MATERIALS AND METHODS

Establishment of the Cell Line

A 32-year-old man was admitted to our hospital in October 1969 with an enlarged left cervical lymph node. After biopsy of the lymph node, Hodgkin's disease of the mixed cellular subtype was diagnosed (Fig 1A and B), and confirmed by two independent pathologists. After extensive examination, the case was identified as clinical stage IV and was successfully treated with combination chemotherapy consisting of prednisone, vinblastine, and cyclophosphamide. Five years later, an abdominal mass, ascites, and bilateral pleural effusion appeared. Cytological examination of the pleural effusion revealed that they were almost all atypical cells, including a number of Hodgkinoid cells (Fig 2A). The patient died 5 days after thoracentesis. At autopsy, mediastinal, paraaortic, and mesenteric lymph nodes were enlarged. Multiple nodules were observed in the lungs, liver, spleen, kidneys, pleura, and peritoneum. Microscopic examination of lymph nodes and nodules showed Hodgkin's disease, lymphocyte depletion subtype (Fig 1C). The cells obtained by the thoracentesis were cultured at a concentration of 1×10^6 cells/mL in RPMI-1640 supplemented with 10% fetal calf serum (FCS), and 40% cell-free supernatant was obtained from the autologous pleural fluid. After 3 days, obvious cell growth was noted, with large floating clusters of cells. The cells were cultured in this condition for 2 weeks. Since then, they have been propagated in continuous culture for >5 years in RPMI 1640 with 10% FCS with a cell-doubling time of ~ 60 hours. The cell line was designated as KM-H2.

Detection of Epstein-Barr Virus

An indirect immunofluorescence test[10] was used for the detection of Epstein-Barr virus-associated nuclear antigen (EBNA).

Chromosome Preparations

Chromosome analysis was performed on KM-H2 cells and the parental pleural effusion cells. Metaphase preparations were obtained after the incubation of 0.05 μg/mL of colcemid, and fixed with methanol acetic acid (3:1). Chromosomes were banded by the trypsin-Giemsa and the Quinacrine staining methods.

Cytochemical Methods

In addition to routine staining with May-Grünwald-Giemsa, the following cytochemical tests were performed on cytocentrifuge preparations: Peroxidase, acid phosphatase,[11] alkaline phosphatase,[12] α-naphthyl acetate esterase,[13] and naphthol AS-D chloroacetate esterase.[13]

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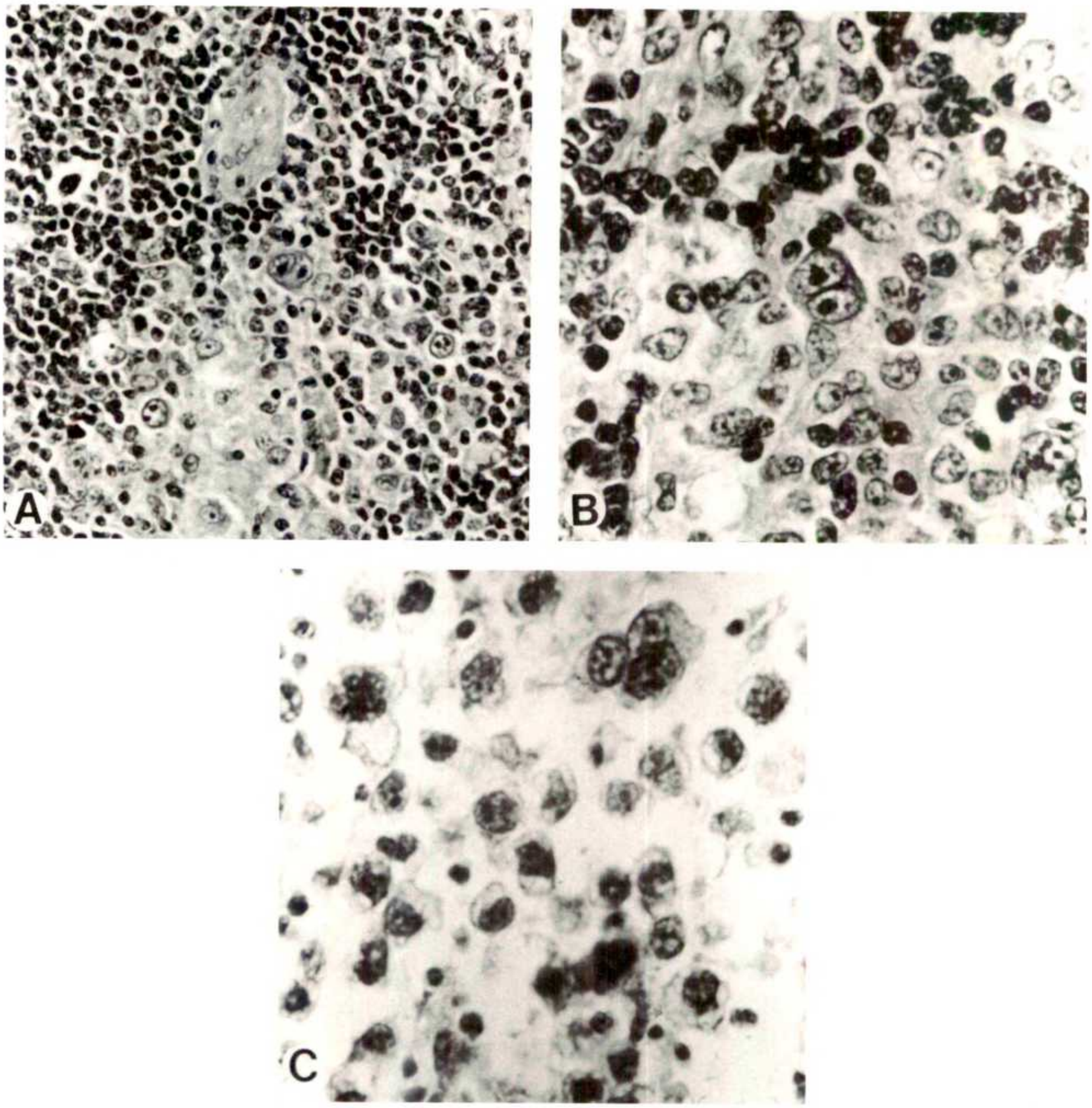

Fig 1. (A) Mixed cell population lying between the interlacing fibroblasts. A classical Sternberg-Reed cell is apparent (H&E ×100). (B) Higher magnification of a typical Sternberg-Reed cell (H&E ×400). (C) Histological appearance at autopsy (H&E ×500).

Studies for Immunologic Markers

Immunoperoxidase methods. Paraffin sections were deparaffinized with xylene and graded alcohol and rehydrated with phosphate-buffered saline (PBS; pH 7.6), as in routine processing. Cytocentrifuged smears were fixed in acetone, at 4 °C, for 10 minutes prior to immunostaining.

The staining procedures using anti-Leu M1 or heteroantisera against lysozyme, α1-antitrypsin, and S100 protein have been described elsewhere[14-16] in detail. The staining procedures with biotin-labeled peanut agglutinin were also performed as previously reported.[17]

Immunofluorescence tests. Cell surface immunoglobulin (Ig) and cytoplasmic Ig were tested by direct immunofluorescence. Antigens defined by mouse monoclonal antibodies were detected by indirect immunofluorescence. Terminal deoxynucleotidyl transferase (TdT) was also stained by indirect immunofluorescence as previously described.[18] The heteroantisera and monoclonal antibodies used in this study are summarized in Table 1. Immunofluorescence was judged under an Olympus fluorescence microscope.

Rosette assays. Cells bearing receptors for sheep erythrocytes,[38] Fc portion of IgG and IgM,[39] and complement[40] were detected as reported previously. The T cell rosette assay was also performed as previously described.

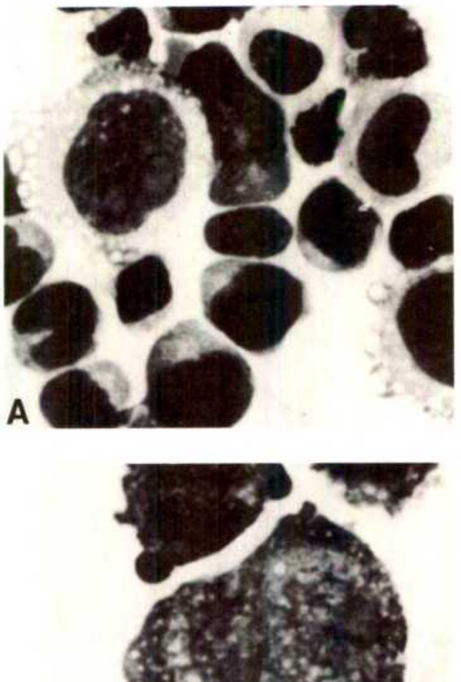

Fig 2. (A) Atypical cells in the original pleural effusion (May-Grünwald-Giemsa, original magnification ×1000; current magnification ×650). (B) A binucleated culture cell with conspicuous nucleoli (May-Grünwald-Giemsa, original magnification ×2,000; current magnification ×1,300).

Phagocytic Activity Tests

The India ink test was performed as previously described.[41] Phagocytic activity of the cell preparations used for the detection of $Fc\gamma$ and C3b receptors was also examined after a 1-hour incubation at 37 °C.

Heterologous Transplantation in Nude Mice

One million viable cells in 100 μL were inoculated into the subcutaneous tissue of 8-week-old mice.

Molecular Genetic Analysis

DNA extraction. Cells were lysed in STE (0.1 mol/L of NaCl, 10 mmol/L of Tris-HCl pH7.4, and 1 mmol/L of EDTA), 500 μg/mL proteinase, and 0.5% sodium dodecyl sulfate (SDS). After incubation, lysate was extracted twice with STE-saturated phenol and then precipitated with ethanol. After treatment with RNase (100 μg/mL) and proteinase K, DNA was re-extracted from the precipitate with STE-saturated phenol and precipitated with ethanol. DNA was dissolved in TE (10 mmol/L of Tris-HCl, pH7.4 and 1mmol/L of EDTA) and stored at 4 °C.

Southern blotting. DNA was digested with *Bam*HI or *Eco*RI, size-fractionated by agarose gel electrophoresis, and transferred onto nitrocellulose paper by the method of Southern.[42]

Preparations of labeled probe DNA. The 3.4-kb *Eco*RI-*Hind*III fragment of the joining region of the heavy chain gene and the 2-kb SacI–SacI fragment of the joining region of the κ chain gene were used as J_H and Jk probes, respectively. The 3-kb *Eco*RI-*Hind*III fragment of the constant region of the T cell receptor β chain was also used as a $C\beta_1$ probe.[43] Nick-translated, ^{32}P-labeled probes were hybridized to nitrocellulose filters in 5 × Denhardt's solution, 0.9 mol/L NaCl, 50 mmol/L of phosphate buffer (pH 7.7), and 5 mmol/L of EDTA, at 65 °C. After hybridization, the washed filters were exposed to film.

RESULTS

Morphological Findings

The KM-H2 cells appeared as clusters of large, nonadherent cells without villi in phase-contrast microscopy. In May-Grünwald-Giemsa-stained cytocentrifuge preparations, these cells had one to three round to oval nuclei with conspicuous nucleoli. Their cytoplasms were stained palely to moderately basophilic and sometimes contained vacuoles (Fig 2B). Their appearance was similar to that of the original pleural effusion cells.

Immunofluorescence Tests for EBNA

The KM-H2 cells were tested in triplicate for EBNA. All observations were negative.

Cytogenetic Findings

The established cell line was composed mainly of cells with 49 to 67 chromosomes, with a minor population having 44, 88, or >100 chromosomes. Karyotypes of KM-H2 cells were highly complex and unstable; however, they were compatible with those of the parental pleural effusion cells, which had near triploid and polyploid sets (Fig 3A). The banded analysis revealed various structural and numerical chromosome aberrations as shown in Fig 3B. The clonal origin of the KM-H2 cell line was ascertained by identifying common marker chromosomes: 5p+, 6p+, and 14p+. Ten years after the initial cultivation, the 5p+, 6p+, and 14p+ chromosomes were still preserved in all KM-H2 cells.

Cytochemical Findings

The original pleural effusion cells were negative for peroxidase reaction.

Enzyme cytochemistry (Table 2) did not demonstrate any peroxidase, alkaline phosphatase, or naphthol AS-D chloroacetate esterase activity in the KM-H2 cells. A weak reaction for acid phosphatase and α-naphthyl acetate esterase was observed in the paranuclear regions of most cells. In a few cells, these reactions were distributed throughout the cytoplasm.

Immunologic Marker Analysis

By immunostaining of the lymph node biopsy from the patient, the SR and H cells were stained distinctly for Leu-M1 and faintly for peanut agglutinin. They were negative for cytoplasmic lysozyme, α1-antitrypsin and S100 protein. Moreover, the KM-H2 cells revealed the same staining pattern as these SR and H cells in situ (Table 3).

Table 1. Polyclonal and Monoclonal Antibodies Used in This Study

Antibody	Specificity/Antigen	Source/Reference
OKIa1 (Mo-Ab)	HLA-DR antigen	Ortho[19]
OKT3 (Mo-Ab)	T cell	Ortho[20]
OKT4 (Mo-Ab)	Helper/inducer T cell	Ortho[20]
OKT6 (Mo-Ab)	Intrathymic T cell; Interdigitating cell	Ortho[20,21]
OKT8 (Mo-Ab)	Suppressor/cytotoxic T cell	Ortho[20]
OKT9 (Mo-Ab)	Transferrin receptor	Ortho[22]
OKT11 (Mo-Ab)	Sheep erythrocyte receptor	Ortho[23]
OKM1 (Mo-Ab)	Monocyte; granulocyte	Ortho[24]
Leu-1 (Mo-Ab)	T cell	Becton Dickinson[25]
Leu-7 (Mo-Ab)	NK cell	Becton Dickinson[26]
Leu-11 (Mo-Ab)	NK cell	Becton Dickinson[27]
Leu-M1 (Mo-Ab)	Monocyte, granulocyte	Becton Dickinson[28]
MCS1 (Mo-Ab)	Granulocyte	Provided by Dr Sagawa[29]
MCS2 (Mo-Ab)	Monocyte, granulocyte	Provided by Dr Sagawa[29]
My4 (Mo-Ab)	Monocyte	Coulter[30]
My7 (Mo-Ab)	Monocyte, granulocyte	Coulter[30]
J5 (Mo-Ab)	Common ALL antigen	Coulter[31]
B1 (Mo-Ab)	B cell	Coulter[32]
Ki-1 (Mo-Ab)	Sternberg-Reed cell	Provided by Dr Stein[33]
R10 (Mo-Ab)	Glycophorin	Provided by Dr Greaves[34]
R4/23 (Mo-Ab)	Dendritic reticulum cell	DAKO[35]
2D1 (Mo-Ab)	Leukocyte	Becton Dickinson[36]
Rabbit anti-TdT Ab	TdT	Provided by Dr Bollum[37]
Rabbit anti-lysozyme Ab	Lysozyme	DAKO
Rabbit anti-α1-antitrypsin Ab	α1-antitrypsin	DAKO
Rabbit anti-S100 protein Ab	S100 protein	Provided by Dr Watanabe[16]
Goat anti-human Ig Ab (F(ab')$_2$ fragment)	Human Ig	Cappel
Goat anti-mouse Ig Ab (F(ab')$_2$ fragment)	Mouse Ig	Cappel

Ig, immunoglobulin; Ab, antibody; Mo-Ab, monoclonal antibody; TdT, terminal deoxynucleotidyl transferase.

As demonstrated in Table 4, the reactivity of the KM-H2 line was almost identical to that of the SR and H cells in the fresh biopsy material reported by others.[45-48] They expressed Ki-1, Leu-M1, HLA-DR antigens and receptors for T cells, transferrin, C3b, and Fc portion of IgG, but lacked markers characterizing B cells (surface Ig, cytoplasmic Ig, B1), T cells (receptors for sheep erythrocytes, Leu-1, OKT3, OKT4, OKT6, OKT8), monocytes (OKM1, MCS2, My4, My7, lysozyme, α1-antitrypsin), dendritic reticulum cells (R4/23), IR cells (OKT6, S100 protein), and NK cells (Leu-7, Leu-11, OKM1). The expression of MCS1 antigens is also compatible with the above results because granulocyte-specific antigens, such as TÜ9, 3C4, and VIM-D5, are detected in freshly obtained SR and H cells.[6]

Phagocytic Activity

The KM-H2 cells did not exhibit phagocytosis of C3b-coated zymosan beads or IgG-coated erythrocytes or India ink particles.

Heterotransplantation in Nude Mice

Subcutaneous inoculation of the KM-H2 line failed to induce tumors in five attempts.

Molecular Genetic Analysis

Figure 4 shows the results of Southern-blot analysis of the immunoglobulin genes and the T cell receptor genes. The J_H segment of *Bam*HI-digested DNA from the KM-H2 cells was observed as a single band at 10-kb, indicating the presence of a heavy chain gene rearrangement; the Jk segment digested by *Bam*HI was detected at 10 kb, however, and was considered as a germline of the κ chain gene. Gene rearrangement of the T cell receptor β chain did not occur in the DNA digested with *Eco*RI or *Bam*HI.

DISCUSSION

In an effort to determine the origin of the SR and H cells in Hodgkin's disease, many investigators have attempted the establishment of permanent cell lines from tissues and body fluids of patients with Hodgkin's disease, but whether the cell lines established derived from SR or H cells seems to be open to question. In fact, several authors[49,50] appear to have erroneously identified the overgrowth of EBV-transformed lymphoblastoid cells as SR and H cells because of their morphologic similarity. Moreover, Harris and colleagues[51] have recently proved that several lines are not related to Hodgkin's disease but are non-human contaminants.

Three other cell lines deserve further discussion. One is the

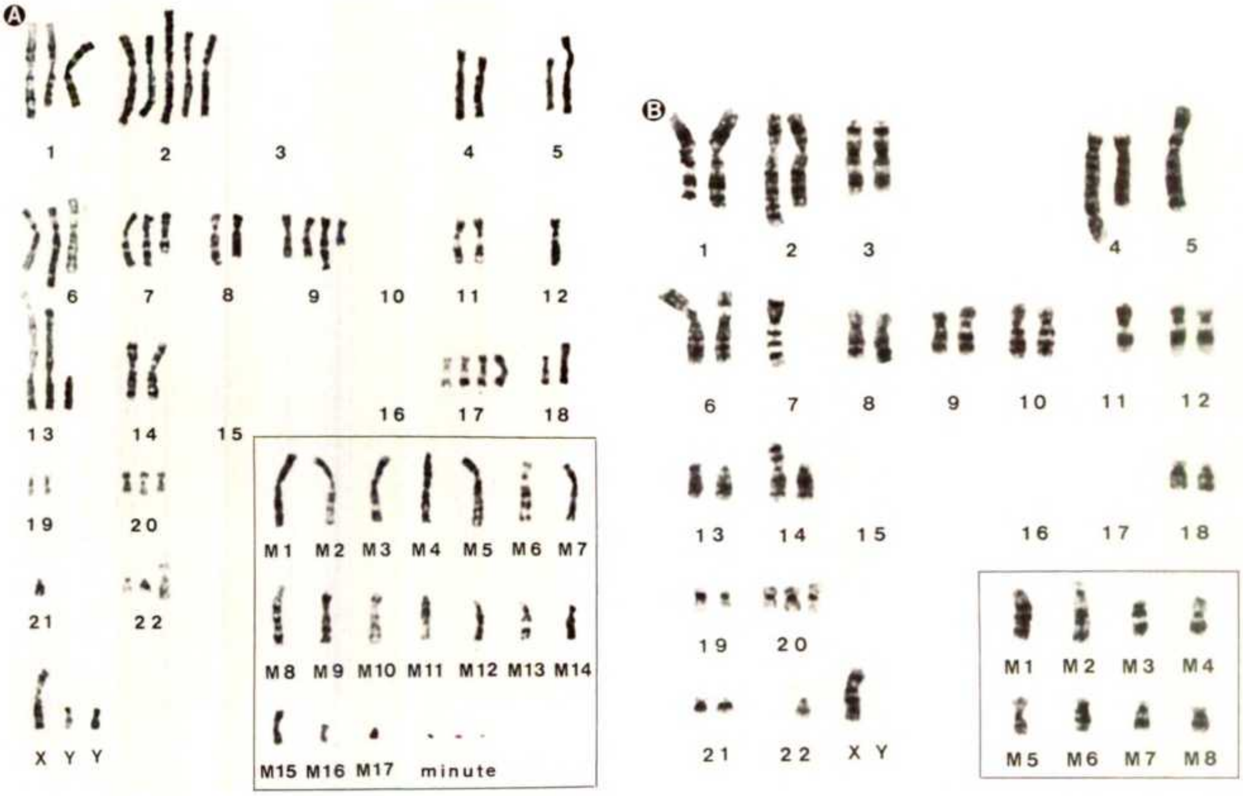

Fig 3. (A) G-banded karyotype of the pleural effusion cell in the triploid range: 70,XY,+Y,−3,−3,−10,−10,−12,−15,−15,−16,−16,+20,−21,+22,i(1q),+1q−,+i(2q),+2×2q−,5p+,6q+,+6p+,+7q−,8q−,+9q+,+9q−,13p+,+13p+,2×14p+,+2×17p+,18p+,+17mar,+3min. (B) G-banded karyotype of a hypodiploid KM-H2 cell in the 50th passage: 44,X,−Y,−5,−7,−11,−15,−15,−16,−16,−17,−17,+20,−22,2q+,4q+,5p+,6p+,7q+,10p+,14p+,+8mar. Unidentified marker chromosome of M1, M3, and M5 appear to be compatible with those of M8, M13, and M15 in (a), respectively. The 5p+, 6p+, and 14p+ chromosomes are preserved in continuously cultured KM-H2 cells for >10 years.

SU/RH-HD-1 cell line described by Olsson and co-workers.[41] The neoplastic nature of this line is apparent from the presence of chromosomal aberrations and the absence of the EBNA reaction. Its human origin is also indicated by the presence of the "alu" family in its DNA. However, the fact that this line does not react with Ki-1 or Leu-M1, which is reported to stain SR and H cells from all cases of Hodgkin's disease, obscures its relation to the SR and H cells.

The other cell line, established by Poppema and colleagues,[52] is the DEV cell line. Their extensive studies clearly indicate this line's neoplastic nature and its close relation to the SR and H cells in the patient's lymph node, but the atypical immunologic phenotype of the original SR and H cells, such as staining with B1 or anti-$\alpha 2$ chain antibody, seems to restrict its importance as a representative of the SR and H cells.

Another cell line, L428, has been reported by Schaadt and co-workers.[9] It was grown from the pleural effusion of a patient with advanced Hodgkin's disease of nodular sclerosing type. Its malignant nature is apparent from multiple structural and numerical chromosome abnormalities associated with a monoclonal pattern of multiple marker chromosomes. In addition, its cytochemical and immunologic features were identical to those of the SR and H cells in the fresh biopsy materials. Therefore, this line appears to be the only one hitherto proved to be derived from the SR and H cells.

Table 2. Cytochemical Findings

	KM-H2 cells	SR and H cells*
Peroxidase	—	—
Acid phosphatase	+	±
Alkaline phosphatase	—	—
α-NAE	+	±
CAE	—	—

α-NAE, α-naphthyl acetate esterase; CAE, naphthol AS-D chloroacetate esterase.

*Data on SR and H cells were taken from previous reports.[9,44]

Table 3. Immunohistochemical Findings

	KM-H2 cells	SR and H cells*
PNA	+	±
Leu-M1	+	+
Lysozyme	—	—
α1-Antitrypsin	—	—
S100 protein	—	—

PNA, peanut agglutinin.

*SR and H cells in the patient's lymph node.

Table 4. A Summary of Immunologic Properties

Property	KM-H2 Cells Positive (%)	SR and H cells*
Sheep erythrocyte receptor	0	–
Fcγ receptor	18	±
Fcu receptor	0	–
C3b receptor	8	±
Receptor for T cells	56	+
Surface immunoglobulin	0	–
Cytoplasmic immunoglobulin	0	(+)?
OKla1	100 (strong)	+
B1	0	±
Leu-1	0	–
OKT3	0	–
OKT4	0 (Weakly positive?)	–
OKT6	0	–
OKT8	0	–
OKT9	91 (Strong)	+
Leu-7	0	Not known
Leu-11	0	Not known
OKM1	0	–
Leu-M1†	+	+
MCS1	48	Not known
MCS2	0	Not known
My4	0	Not known
My7	0	Not known
R10	0	–
R4/23	0 (Weakly positive?)	–
Ki-1	96	+
J5	0	Not known
2D1	0	–
Peanut agglutinin†	+	±
Lysozyme†	–	–
α1-antitrypsin†	–	±
S100 protein†	–	–

*Data for SR and H cells are taken from previous reports.[45-48]

†Tested by the immunoperoxidase method.

Our KM-H2 cell line was established from the pleural fluid of a patient whose diagnosis was histologically confirmed by two independent pathologists. This diagnosis was supported by the finding that the "SR and H cells" in the patient's lymph node were distinctly positive for Leu-M1.[14] This line's neoplastic nature was indicated by the presence of structural and numerical chromosome aberrations and by the absence of the EBNA reaction, although tumor formation after subcutaneous inoculation of KM-H2 cells was not observed. Moreover, the KM-H2 cells showed the same immunostaining pattern as the SR and H cells in the patient's lymph node and shared nine positive properties and a number of negative properties with the SR and H cells in the fresh biopsy materials reported by other authors[44-48] (Tables 2 and 4). The cells also retained some of the chromosomal abnormalities observed in the pleural effusion cells. Thus, it seems reasonable to conclude that the KM-H2 cell line originated from the SR and H cells of Hodgkin's disease.

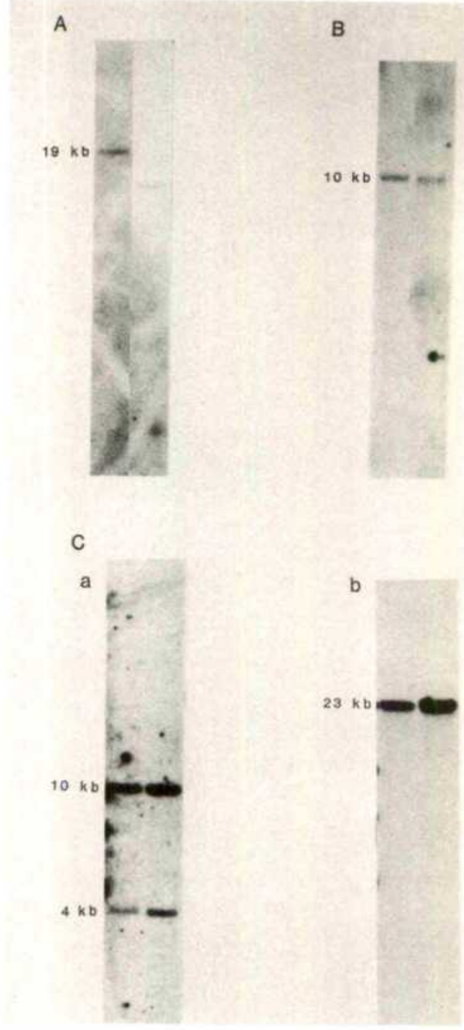

Fig 4. Southern-blot analyses of KM-H2 cells with Ig J_H (A), Ig Jk (B) and T cell receptor Cβ1 (C) as probes. In Figs 1 through 4, left lanes are controls: (A, B) T cell line MT-2; (C) B cell line BALL-1. Right lanes are KM-H2. (A) Germline Ig J_H segment is represented by 19 kb when digested with *Bam*HI; (B) germline Ig Jk segment is shown by 10 kb when cut with *Bam*HI. (C) (a) Ten kilobases and 4 kb correspond to the germline T cell β chain constant region genes, Cβ1 and Cβ2, respectively, when digested with *Eco*RI; (b) 23 kb represents the germline configuration of T cell receptor β chain gene, including Cβ1 and Cβ2 when cut with *Bam*HI.

Recent advances in molecular biology have made it clear that somatic recombinations of the immunoglobulin genes occur in B cell ontogeny. Korsmeyer and co-workers[53,54] proposed a model of genetic maturation in human B cell lineage and revealed that analysis of the immunoglobulin gene rearrangement was useful to determine the origin of leukemias and lymphomas.[55] Recently, several authors[56,57] have also reported that the T cell antigen receptor genes have a genomic structure similar to that of the immunoglobulin genes and that somatic recombinations of these genes take place in T cell differentiation. Because T cell receptor gene rearrangement is expected to occur only in T cell lineage, the analysis of this gene appears useful to identify the cellular origin of hematopoietic tumors.[58,59] Therefore, we used these molecular genetic approaches to analyze the cellular origin of the KM-H2 line, which is still obscure in spite of extensive cytochemical and immunologic studies.

The J_H region of the immunoglobulin genes in this line was rearranged, but not the Jk region or the constant region of the T cell receptor β chain genes. The germline configuration of the T cell receptor β chain genes, as well as the lack of the T cell antigens, virtually excludes the possibility of its T cell origin. However, the presence of only J_H recombination is not sufficient to conclude that it is of B cell lineage, since such a genomic pattern is also observed in some myelogenous leukemia cells.[60,61]

Indeed, the following observations favor its origin from the nonlymphoid cell lineage rather than from the B cell lineage, in contrast to recent reports[52,62] that support the B cell origin for the nodular sclerosing subtype of Hodgkin's disease: (a) The expression of common ALL antigens or TdT, which is usually observed at this stage of B cell differentiation, could not be detected in the KM-H2 cells; (b) neither cytoplasmic Ig nor B1 antigens could be induced by the presence of 4β-phorbol-12-myristate-13 acetate (TPA) (unpublished observations); (c) Leu-M1 and MCS1 antigens were expressed, and (d) a reaction for acid phosphatase and α-naphthyl acetate esterase was observed in most cells. Therefore, our results suggest the heterogenous cellular origin of Hodgkin's disease, especially among the histological subtypes, although further studies are needed.

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α-Interferon Activates the Natural Killer System in Patients With Hairy Cell Leukemia

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To elucidate the mechanisms of α-interferon's (α-INF) therapeutic effect on clinical and laboratory findings in hairy cell leukemia, we sequentially monitored different immunologic parameters in three patients treated with recombinant α-INF. The most evident effect of this treatment on the immune system was the recovery of natural killer (NK) cell in vitro activity of peripheral blood lymphocytes, which was severely impaired before therapy. In particular, NK function began to improve after 3 months, and a complete recovery was obtained after 6 months in all cases. This increase parallels the improvement in clinical and laboratory findings.

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HAIRY CELL LEUKEMIA (HCL) is a malignant B cell disorder usually characterized by isolated splenomegaly and peripheral pancytopenia associated with the presence in the blood of mononuclear hairy cells that infiltrate the spleen and bone marrow.[1,2] Although splenectomy has been the treatment of choice in this disease for a number of years, a large number of patients fail to respond to this approach.[2] The use of different therapeutic strategies based on chemotherapeutic agents has been unsatisfactory.[3] A highly effective treatment of these patients has been recently offered by α-interferon (α-INF)[4-7]; however, the mechanisms that account for its therapeutic efficacy are unclear. In particular, no information has been provided thus far on the possible modifications of immunologic parameters induced by α-INF treatment, which may correlate with the improved clinical and laboratory findings in HCL-treated patients.

Interferons are powerful stimulants of natural killer (NK) cell activity,[8] and this seems to be the mechanism of INF antitumor action in the animal model.[9] On the other hand, severe deficiency of NK function has been demonstrated in HCL-untreated patients.[10,11] The aim of this study was to monitor different immunologic parameters sequentially in HCL patients treated with recombinant α-INF (rINF-α_2). Special attention was paid to the phenotypic and functional analysis of NK cells, and these findings have been related to the modifications observed on the clinical and laboratory parameters. A 9-month follow-up was taken into account.

MATERIALS AND METHODS

Patients. Three patients (two males and one female aged 64, 44, and 64 years, respectively) with HCL were treated with rINF-α_2 obtained from the Schering Corp (Shering, Italy) as lyophilized powder (5×10^6 IU/vial) and reconstituted with sterile water. Informed consent was obtained from all patients, who had been advised of procedures and attendant risks. The diagnosis of HCL was based on clinical, morphologic, and cytochemical criteria[1,2]; none of the patients had a splenectomy or previous treatment. Before rINF-α_2 therapy, a severe neutropenia ($<500/\mu L$) was present in all patients; two of them also had thrombocytopenia and mild anemia (Fig 1). The treatment started in Nov 1984 and consisted of 2×10^6 IU/m^2 of rINF-α_2 injected subcutaneously three times a week. Adverse reactions of moderate intensity consisting mainly of fever that was controlled by acetaminophen were seen in two patients. A severe neutropenia with septic fever developed in one patient during the fourth week of treatment, requiring interruption of rINF-α_2 administration for 40 days.

Separation procedures. Mononuclear cells were obtained from freshly drawn heparinized peripheral blood by centrifugation on Ficoll/Hypaque gradient. The cells were washed three times and resuspended in RPMI 1640 (GIBCO, Grand Island, NY). Adherent cells were removed by two sequential incubations for 45 minutes in plastic Petri dishes at 37 °C in an atmosphere of 95% air and 5% CO_2.

Phenotypic analysis. The following monoclonal antibodies (MoAbs) were used in this study: OKT3 (Ortho Pharmaceutical, Raritan, NJ) reacts with mature T cells[12]; OKT4 and OKT8 (Ortho) selectively bind to T lymphocyte subsets including cells with helper and suppressor/cytotoxic activity, respectively[12]; HNK-1 (Leu-7, Becton-Dickinson, Sunnyvale, Calif) defines a differentiation antigen expressed on NK and K cells[13]; and anti-Tac (kindly provided by Dr T. Uchiyama, Kyoto, Japan) has been found to recognize the membrane receptor for interleukin 2 on T and B cells[14] and on hairy cells.[15]

Nonadherent cells obtained as described were incubated for 30 minutes at 4 °C with the aforementioned reagents. Following incubation, cells were washed three times, and a fluorescein-conjugated F(ab)$_2$ goat antimouse immunoglobulin antiserum (Cappel Laboratories, Cochranville, Pa) was added for 30 minutes at 4 °C. After further washing, 300 cells were examined under a Leitz Orthoplan microscope (Wetzlar, FRG) equipped for epifluorescence.

Enzyme histochemistry and immunohistology of bone marrow biopsies were performed according to the method previously described in detail by using a series of markers, including tartrate-resistant acid phophatase, RFB-4, anti-Tac, and anti-HLA-DR MoAbs.[16]

Functional evaluations. NK activity was assessed by lysis of ^{51}Cr-labeled K-562 target cells as previously described.[17] Briefly, target cells were labeled overnight at 37 °C in 5% CO_2 with 100 μCi $Na^{51}CrO_4$ (CEA IRE Sorin, Biomedica, Saluggia, Italy), extensively washed three times before use, and then adjusted to a final concentration of 10^5 cells/mL in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Microbiological Products, Wakersville, Mass). To perform the test, 100 μL of labeled targets (10^4 cells) were cocultured with effector cells for four hours at 37 °C in 5% CO_2 at different dilutions to final ratios between effectors and target cells (E/T): 5:1, 10:1, 20:1, 40:1, and 80:1 in triplicate round-bottom wells (Limbro plates Limbro, Hamden, Conn). Triplicate wells with

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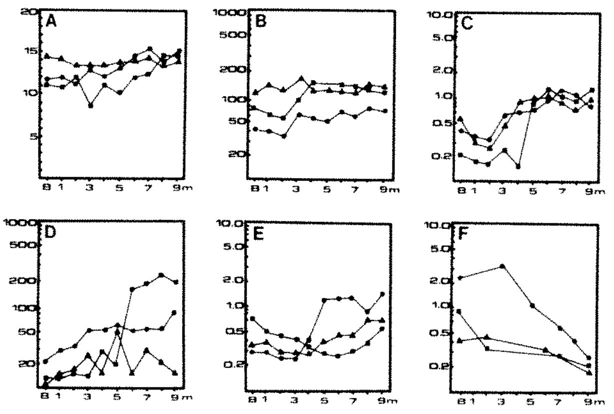

Fig 1. Peripheral blood findings before (B) rINF-α_2 therapy and during the follow-up at 1, 2, 3, 4, 5, 6, 7, 8, and 9 months (m). ▲, ●, and ■ represent individual patients (F.N., M.R., and P.A., respectively). (A) Hemoglobin (gr/dL), (B) Platelets (× 10^{-3}), (C) Neutrophils (× 10^{-3}), (D) Monocytes, (E) Lymphocytes (× 10^{-3}), (F) Hairy Cells (× 10^{-3}).

target cells in medium alone and in detergent were assessed to determine spontaneous and maximum release, respectively. Following this incubation supernatants were harvested and counted in a gamma counter. The mean value of triplicate assays was used to calculate the percentage of cytotoxicity according to the formula: percent cytotoxicity = (cpm release in test − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release) × 100. Spontaneous release from the target cells was always less than 8%.

The proliferative response to phytohemoagglutinin (PHA) was evaluated as previously described.[17]

Twenty age-matched healthy volunteers were used as controls. All data are expressed as the mean ± SE of the mean. Statistical analyses were performed by the Cochran-Cox test.

RESULTS

Figure 1 shows the changes in peripheral blood counts in individual patients following rINF-α_2 treatment. Two patients had an improvement of their anemia and thrombocytopenia. With regard to WBC, the neutropenia dramatically improved in all cases, and the number of monocytes showed a net increase in two cases (M.R., P.A.). Lymphocytes increased in two out of three patients (M.R., F.N.). The number of hairy cells in the peripheral blood, as determined by morphology both on May-Grunwald-Giemsa–stained smears and phase-contrast examination of cells in suspensions, decreased in all patients.

Bone marrow biopsies performed after 3 and 7 months of rINF-α_2 therapy revealed an increase of the myeloid component in all patients. A marked reduction of hairy cell infiltration was observed in one case (P.A., from 90% to 20%), whereas in the two other cases, the frequency of hairy cells, although diminished, continued to represent a consistent proportion of the bone marrow cells (70% to 80%).

The evaluation of immunologic markers (Fig 2) shows a slight but not significant decrease of the T4/T8 ratio. Although the frequency of cells with an NK-related phenotype, as determined by the HNK-1 MoAb, increased in all patients (Fig 2), the absolute numbers of these cells were consistently within the normal range (<390/µL) in all cases.

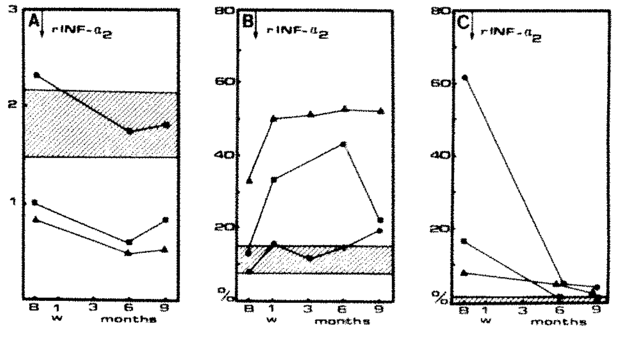

Fig 2. Phenotypic analysis of peripheral blood mononuclear cells with MoAbs before (B) and after 1 week ($\underset{w}{1}$) or 3, 6, and 9 months of rINF-α_2 therapy. Shaded areas represent the range values for controls. ▲, ●, and ■ represent individual patients (F.N., M.R., and P.A., respectively). (A) T4/T8 Ratio, (B) HNK-1 Positive Cells, (C) Anti-TAC Positive Cells.

The percentage of Tac-positive cells was reduced in all patients according to the decrease of the number of hairy cells.

Analysis of NK in vitro function (Fig 3) demonstrated an initial decrease, at different E:T ratios, following the first months of rINF-α_2 therapy. NK function began to increase after 3 months, and complete recovery was observed after 6 months in all cases. At the 9-month evaluation, results were comparable with those obtained at the 6-month evaluation. Interestingly, the best response was observed in patient M.R. whose cells expressed the highest frequency of Tac$^+$ cells before treatment.

PHA-induced blastogenesis was reduced with respect to controls and did not reveal significant differences during the follow-up of patients.

DISCUSSION

We have demonstrated that the treatment of HCL with rINF-α_2 restores the NK cell system, which is severely depressed in these patients before α-INF therapy. This parallels the improved clinical and laboratory findings.

α-INF belongs to a large family of biologic response-modifying drugs whose activity includes an antiviral effect, a direct cytotoxic property, and a cell differentiation function.

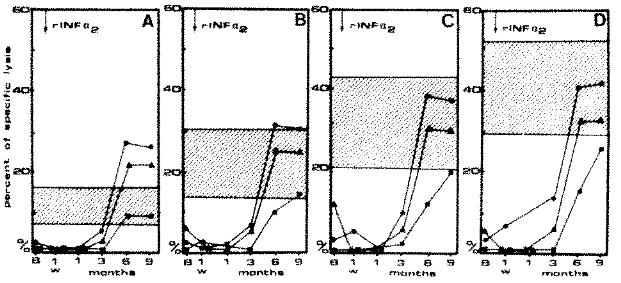

Fig 3. NK in vitro activity before (B) the rINF-α_2 activity and during the follow-up at 1 week ($\underset{w}{1}$) and 1, 3, 6, and 9 months. Shaded areas represent the range values for controls. ▲, ●, and ■ represent individual patients (F.N., M.R., and P.A., respectively). (A) 10:1 E:T Ratio, (B) 20:1 E:T Ratio, (C) 40:1 E:T Ratio, (D) 80:1 E:T Ratio.

α-INF has been used in several neoplastic disorders,[18,19] and as a matter of fact, the best response has been observed in HCL patients.[20] However, no complete and longitudinal studies are available that document the effects of α-INF therapy on different immunologic parameters in this disease. For one thing, our data confirmed previous studies demonstrating that the NK activity in these patients is impaired.[10,11] Since the defect of NK function persisted even when these cells were isolated from other cell populations (ref 11 and our unpublished results), the impairment of cytotoxic function cannot be consequent to cellular dilution. Following rINF-α_2 therapy, the NK function initially decreased, but after 3 months the values began to improve and definitely recovered after 6 months.

NK cells have been thought to play a significant role in the host defense mechanisms involved in the resolution of certain infectious agents and in tumor rejection.[21] Furthermore, several lines of evidence demonstrate that NK cells can directly lyse tumor cells,[22] especially following interferon activation,[23] and can inhibit clonogenic growth of fresh leukemic cells.[24] A lack of production of endogenous α-INF in HCL, possibly resulting from chromosomal abnormalities,[4] could be involved in the defective NK function. Therapy with rINF-α_2 could restore a mechanism that currently takes place normally in the host's defense against foreign insults and/or in the control of tumor growth.

Since interleukin 2 (IL 2) activates NK cells,[25] the severe deficency of NK activity reported in untreated HCL patients[10,11] could also be related to the impaired levels of IL 2 consequent to its absorbtion by hairy cells, which are equipped with IL 2 receptors.[15] In line with this interpretation, other neoplasms, ie, cutaneous T cell lymphomas usually characterized by the expression of IL 2 receptors,[26] are highly responsive to α-INF.[27] The two mechanisms mediated by IL 2 and α-INF might synergistically act on NK boosting.[28] Consistent with this latter interpretation, we found that before starting the rINF-α_2 therapy the incubation at 37 °C for 18 hours of peripheral blood cells from our patients both with rINF-α_2 and with recombinant IL 2 led to an enhancement of the NK in vitro function (data not shown).

The attribution of α-INF effects against tumor growth solely to its enhancement of NK function would be inappropriate. In fact, apart from the activation of NK cells, other mechanisms may be involved in patients affected by different disorders following α-INF treatment.[29] As a matter of fact, the association of other effects could account for the lack of correlation usually observed between clinical improvement and evaluation of the NK in vitro activity in patients other than those with HCL.[30-32] Differences in the type of α-INF, its schedule or route of administration, and especially the dose used could also be a source of discrepancy. In this regard, it has been recently demonstrated[33] that high doses of INF display a direct activity (ie, a cytopathic effect), whereas low doses increase the indirect power of this substance through an enhancement of the antitumor activity of the immune system (eg, the effectiveness of NK cell activation).[34-35] In this regard, evidence recently provided that α-INF enhances the expression of class II HLA antigens on hairy cells has led to speculation that α-INF might exert its antileukemic activity by potentiating a cytotoxic mechanism.[36] The good correlation between the clinical improvement and the NK in vitro function observed in our study suggests that the NK system plays a relevant role in α-INF–treated HCL patients. Thus, in some diseases (notably HCL) the activation of NK activity may be prominent, whereas in other disorders, the cytopathic effect may be the main mechanism involved in tumor growth control. The latter has been clearly demonstrated in some neoplastic conditions, especially using high doses of chemotherapy.[33]

Another effect of α-INF is its cell differentiation property.[37] In this regard, the improvement of neutropenia and bone marrow findings in HCL patients could be consequent to an effect on cellular differentiation either directly[38] or via different circuits involving activated cells.[39] This differentiation ability could also account for the increase in the monocytic component observed in two of our patients, similar to the results described by Territo et al[40] in cancer patients.

We conclude that α-INF therapy leads to a series of effects. Among these, at least in HCL patients, the improvement of NK cell activity might be crucial. Of course, the demonstration of a specific lysis of hairy cells by autologous cytotoxic cells will represent the final proof supporting the relevance of cytotoxic mechanisms in α-INF–treated HCL patients. The small number of patients and the short follow-up preclude firm statements regarding the impact of NK evaluation on the prognosis of these patients. We only want to emphasize that the analysis of immunologic parameters might help to clarify the possible therapeutic action of α-INF in HCL. Further studies on a large series of patients are needed to clarify whether evaluation of NK activity may be useful in predicting the prognosis and/or monitoring α-INF therapy by determining the optimal dose, schedule, and duration of treatment.

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Response to 2′-Deoxycoformycin After Failure of Interferon-α in Nonsplenectomized Patients With Hairy Cell Leukemia

By Kenneth A. Foon, Gordon M. Nakano, Charles A. Koller, Dan L. Longo, and Ronald G. Steis

Two patients with hairy cell leukemia with massive splenomegaly and severe pancytopenia were treated with recombinant α-A interferon (IFN-α-2a). There was no significant response to a trial of IFN-α-2a (11 and 20 weeks) with respect to blood counts or spleen size. Subsequent treatment with 2′-deoxycoformycin (dCF) for 8 consecutive weeks (4 mg/m^2/wk) resulted in normalization of spleen size and a normalization of peripheral blood counts and bone marrow in one patient. The second patient demonstrated a reduction in spleen size and improved blood counts following 9 weeks of dCF therapy but eventually became refractory. This demonstrates that dCF is non-cross-resistant with interferon and confirms the efficacy of dCF in nonsplenectomized patients.

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HAIRY CELL leukemia is a lymphoproliferative disorder generally involving B lymphocytes and usually presents with cytopenias and splenomegaly.[1,2] Standard initial therapy has been splenectomy, which often restores hematologic parameters to normal.[3-5] Unfortunately, most of these patients relapse with recurrent cytopenias weeks to years following splenectomy. Treatment with cytotoxic agents such as chlorambucil may result in a temporary reduction in tumor burden but are generally not very effective in improving granulocyte counts.[6] Recently, in a preliminary study using partially purified natural interferon-α at a dose of 3 × 10^6 units daily, three complete remissions and four partial remissions were observed in seven patients with hairy cell leukemia.[7] All seven patients had a recovery of peripheral blood counts. We and others have reported similar responses to recombinant interferon-α, with greater than 90% response rates.[8-10] Since most patients receiving interferon-α to date have had prior splenectomy, the clinical efficacy of interferon-α in nonsplenectomized patients remains uncertain.

Even more recently, the lymphocytotoxic agent, 2′-deoxycoformycin (dCF), or pentostatin, was reported to have induced rapid, complete remissions in two previously untreated, nonsplenectomized patients with hairy cell leukemia.[11] dCF is a tight-binding inhibitor of adenosine deaminase (ADA), the enzyme responsible for the conversion of adenosine to inosine and deoxyadenosine to deoxyinosine. In the absence of ADA activity, deoxyadenosine is phosphorylated to deoxyadenosine triphosphate (dATP) rather than deaminated to deoxyinosine, which may contribute to the lymphocytotoxicity.[12] The biochemical basis for this toxicity remains controversial, and recent studies have suggested that the principal cause of cell death in normal resting human lymphocytes is depletion of NAD.[13] The drug has been used successfully in the treatment of acute lymphoblastic leukemia, but has been associated with severe toxicity when used in higher doses.[14] Clinical nonlymphoid toxicity has been correlated with depletion of ATP[15] and with accumulation of dATP in red cells.[16,17] Further confirmation of the promising results with dCF in patients with hairy cell leukemia as well as the apparent lack of toxicity is needed, and the comparative efficacy of interferon-α *v* dCF in this disease remains to be demonstrated. We report the results of dCF therapy in two nonsplenectomized hairy cell leukemia patients who had failed prior therapy with recombinant α-A interferon (IFN-α-2a).

MATERIALS AND METHODS

Patient 1. The first patient was a 42-year-old Romanian male who was in apparent good health until March 1984 when he noted the insidious onset of fatigue, weight loss, and exertional dyspnea. These symptoms progressed, and in Aug 1984, he was admitted to the University of Michigan Medical Center with acute epistaxis.

Physical examination was significant for moderate epistaxis without an obvious source. The liver was enlarged with a 24-cm span, and the spleen was palpable 12 cm below the left costal margin. The remainder of his examination was unremarkable.

Initial laboratory data included a hemoglobin level of 4.9 g/100 mL, hematocrit value of 15.7%, and a platelet count of 41 × 10^9/L. The white blood count was 4.3 × 10^9/L with 14% neutrophils, 3% bands, 81% lymphocytes, 2% monocytes, plus 13 nucleated red cells. Most of the lymphoid mononuclear cells were noted to have bean-shaped nuclei and ragged cytoplasmic margins typical of hairy cells. Tartrate-resistant acid phosphatase stain was positive, and bone marrow biopsy results confirmed the diagnosis of hairy cell leukemia.

The epistaxis was controlled with local packing and transfusion of random donor platelets and packed red cells. Splenectomy was strongly recommended but was refused by the patient.

For the next month, the patient was treated supportively with platelets and packed red cells for recurrent epistaxis as well as with multiple antibiotics for episodes of fever. He continued to refuse splenectomy on repeated occasions.

In Sept 1984, the patient was referred to the Biological Response Modifiers Program of the National Cancer Institute. Following the patient's informed consent, he underwent a 12-week trial of IFN-α-2a (Hoffmann-La Roche Inc, Nutley, NJ) administered on a daily basis subcutaneously at a dose of 3 × 10^6 U/d from Sept 24 through Dec 11, 1984. Although during this period his epistaxis subsided and his transfusion requirements appeared to decrease somewhat, his severe pancytopenia and massive hepatosplenomegaly persisted. Due to a falling platelet count with recurrent petechiae and epistaxis, the interferon therapy was discontinued.

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Six weeks after stopping IFN-α-2a therapy and following the patient's informed consent, he began to receive dCF at a dose of 4 mg/m^2/wk as an intravenous (IV) bolus followed by a liter of normal saline infused for a period of one to two hours. Allopurinol, 300 mg/d orally, was also given during the first week.

Patient 2. The second patient was a 65-year-old Pakistani woman who had had a 15-year history of splenomegaly ultimately diagnosed as hairy cell leukemia in 1979. She was treated with a number of courses of cytotoxic chemotherapy (chlorambucil and cyclophosphamide) but was not considered a good surgical candidate. She was referred to the National Cancer Institute for IFN-α-2a therapy. Physical examination was significant for a massively enlarged spleen (approximately 30 cm below the left costal margin). Her liver was palpable 9 cm below her right costal margin.

Initial laboratory data revealed a hemoglobin level of 11.5 g/100 mL, hematocrit value of 35%, and platelet count of 92 × 10^9/L. The WBC count was 133 × 10^9/L with 2% neutrophils, 0% bands, 0% monocytes, and 98% lymphocytes having the characteristic appearance of hairy cell leukemia cells. Tartrate-resistant acid phosphatase stain was positive, and bone marrow biopsy results confirmed the diagnosis of hairy cell leukemia.

On Dec 17, 1984, the patient began a daily dose of 3 × 10^6 units of rIFN-αA (informed consent was obtained prior to therapy). There were no changes in any of the laboratory or physical parameters throughout the course of 20 weeks of therapy. She was subsequently treated with dCF at a dose of 4 mg/m^2/wk as previously described (informed consent was obtained prior to therapy). Allopurinol therapy, 300 mg/m^2 orally, was also begun.

Measurement of intracellular adenine nucleotides. Red cell ATP and dATP were measured using previously reported techniques.[16]

Criteria for response. Complete remission was defined as the absence of hairy cells in the bone marrow aspirate and biopsy specimen, bone marrow granulocytes above 35%, and recovery of the hemoglobin level to $\geq$12 g/dL, the absolute granulocyte counts to $\geqq$1.5 × 10^9/L, and the platelet counts to $\geq$100 × 10^9/L for $\geqq$1 month. Partial remission was defined as a decrease of the hairy cell leukemia infiltrate by more than 50% from pretreatment levels and bone marrow granulocytes above 35%, with recovery of peripheral blood parameters as defined for complete remission for $\geqq$1 month. Minor remission was defined as a decrease of the hairy cell leukemia infiltrate by <50% from pretreatment values, bone marrow granulocytes of 25% to 35%, and improvement of peripheral blood parameters for $\geqq$1 month. Stabilization was defined as stabilization of all detectable abnormalities of bone marrow, peripheral blood, and physical examination. Progression was defined as the appearance of new, or worsening of preexistent abnormalities of peripheral blood, bone marrow, or physical examination. The duration of response was measured in terms of the number of days from the initial documentation of response to the documentation of progression.

RESULTS

No subjective or objective toxicity was observed during treatment with dCF in the first patient. Within 1 week after receiving his first dose of dCF, improvement was seen, with a decrease in liver and spleen size and reduction in circulating hairy cells. The platelet count and hepatosplenomegaly continued to improve progressively, and both parameters were within normal limits by week 8 of treatment (Fig 1). Also, by the eighth week of treatment, the absolute granulocyte count rose to 1 × 10^9/L (gradually rose to 2.6 × 10^9/L), and there were no longer visible hairy cells or nucleated erythrocytes on the peripheral blood film. Although he had no toxicity, the patient refused further therapy after week 8 because he was feeling well. Despite no further therapy, the patient's hematocrit value continued to improve and by week 16 was 41%. A bone marrow biopsy specimen showed normal hematopoietic cells without hairy cells. Red cell ATP remained within normal limits (135 to 156 [picomoles] pmol/10^6 cells), and red cell dATP rose no higher than 8.9 pmol/10^6 cells during treatment with dCF (data not shown).

The patient was lost to follow-up, but 7 months later agreed to return for an examination and blood count; he refused a bone marrow aspiration and biopsy. His hemoglobin level was 15 g/dL; hematocrit value, 45%; platelets, 114 × 10^9/L; and WBC count, 3.6 × 10^9/L (60% neutrophils, 10% bands, 29% lymphocytes, 1% monocytes). The peripheral blood smear revealed no hairy cell leukemia cells, but some very large abnormal appearing platelets were noted. His spleen was not palpable, and he had no physical complaints.

Patient 2 did not have a dramatic response to weekly injections of dCF but did have a rapid reduction in the circulating hairy cells from 179 × 10^9/L to 23 × 10^9/L during her ninth week of therapy, with improvement in her hematocrit value from 28% to 36% and stable platelet counts. Her spleen decreased in size from 30 cm to 16 cm below the left costal margin. A skin rash and keratoconjunctivitis developed in week 6, leading to a reduction of therapy to 4 mg/m^2 every other week for the next 12 weeks. Following this therapy, her white count increased, and she was given 4 mg/m^2 of dCF on two successive days. Her WBC count continued to rise despite stable hematocrit and platelets counts, and she was switched to chlorambucil treatment. She

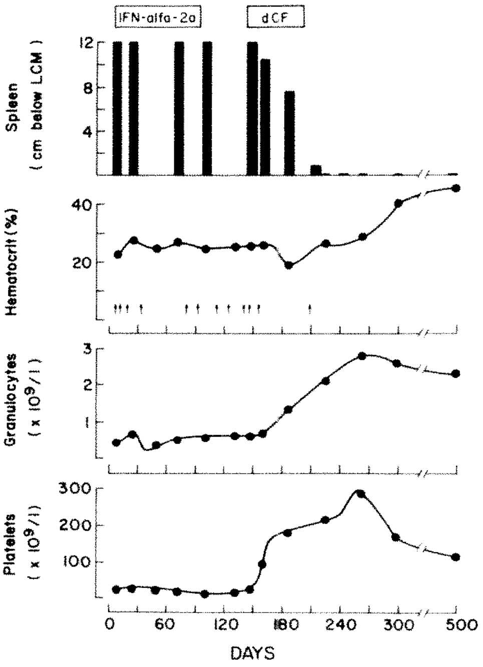

Fig 1. Clinical and laboratory parameters in patient 1 during and following interferon and dCF therapy. Arrows represent RBC transfusions. LCM, left costal margin.

did not respond to chlorambucil therapy and eventually was taken to surgery for a splenectomy. She had a transient minor response to splenectomy.

DISCUSSION

Within the past 2 years, two new therapeutic agents have shown exceptional promise for the treatment of hairy cell leukemia. Both interferon-α and dCF have been reported to induce complete remissions in this disease, with early results appearing far superior to conventional chemotherapeutic agents such as chlorambucil. Treatment-related toxicity has also been minor. Given the small numbers of patients reported, however, with only short-term observation, the relative efficacy of each of these agents remains uncertain with respect to extent of disease and prior therapy.

Neither of our patients had prior splenectomy. They received IFN-α-2a (11 and 20 consecutive weeks, respectively) but had no clear response to this therapy. Although these two patients may have eventually responded to interferon at the same dose or at increased doses, it was decided to discontinue the treatment because of progressive thrombocytopenia with fresh bleeding and petechiae in one patient and painful splenic infarcts in the other. Both patients were subsequently treated with dCF, a second investigational drug. After only eight weekly injections of dCF the first patient had a dramatic response, with normalization of peripheral counts and bone marrow and resolution of severe hepatosplenomegaly. Seven months later he had no hepatosplenomegaly and a normal hematocrit value. However, he had moderate leukopenia and thrombocytopenia suggestive of recurrent hairy cell leukemia in his bone marrow. Unfortunately, he refused a bone marrow aspiration and biopsy. The other patient had a transient improvement in blood counts and reduction in the size of her spleen. Preliminary data from Spiers and co-workers have confirmed the effectiveness of dCF in nonsplenectomized patients with hairy cell leukemia as well as its effectiveness after failure of interferon-α.[18]

The failure of these two patients to respond to IFN-α-2a, contrary to the extremely high response rates in most patients, cannot be accounted for with certainty. Given the low efficacy of other forms of adoptive or active immunotherapy in the presence of high tumor burdens in laboratory animal models, it is possible that these patients might have responded if they had undergone prior splenectomy or if treatment had been started earlier in the course of the disease. Suboptimal drug delivery to tumor sites or an intrinsic resistance of the patient's hairy cells to IFN-α-2a is also possible.

The optimum dose and frequency of dCF administration remains to be established. In phase I studies in acute leukemia, daily IV administration of dCF for three to seven consecutive days in doses of 0.15 to 1.0 mg/kg/d resulted in a fairly high incidence of gastrointestinal toxicity (nausea, vomiting, and diarrhea), with occasional keratoconjunctivitis nephrotoxicity, hepatotoxicity, CNS toxicity, or death.[16] The two hairy cell leukemia patients with complete responses to dCF reported by Spiers et al[11] initially received two or three consecutive daily IV boluses of dCF at a dose of 5 mg/m^2 followed by two consecutive days of treatment every 2 weeks for a total of 15 or 16 doses. Reported toxicity to lower doses of dCF was moderately severe nausea and vomiting in one patient and a transient photosensitivity rash in both patients. Our patients were treated with a lower weekly dose of 4 mg/m^2 without a loading dose. Despite the massive tumor burden, the rapidity of antitumor effect was nonetheless striking, and there was no demonstrable toxicity in one patient. The minimal increase in red cell dATP and decrease in ATP seen in this patient was consistent with the observed lack of toxicity.[17] The second patient did not have a dramatic response to dCF, and her therapy was complicated by rash and keratoconjunctivitis. Unfortunately, we did not measure red cell adenine nucleotides in this patient. The treatment schedule chosen for our patients as induction therapy thus appears to be a reasonable one with respect to safety and efficacy, although the long-term toxicity of dCF in hairy cell leukemia patients remains to be studied. Toxicity in patient 2 was improved when therapy was decreased to every other week.

In summary, we report one complete response and one minor response to dCF in two nonsplenectomized hairy cell leukemia patients following their failure to respond to IFN-α-2a. Weekly outpatient low-dose dCF therapy without a loading schedule was extremely well tolerated. These cases confirm the responsiveness of hairy cell leukemia to dCF in nonsplenectomized patients with high tumor burdens and suggest a possible superiority of dCF over interferon-α in this setting as well as a lack of cross-resistance with interferon. Unanswered questions still remaining include remission duration and survival in responders to interferon-α and dCF and the role of maintenance therapy using either interferon-α or dCF. Although the dramatic resolution of splenomegaly in patient 1 with dCF raises the question of whether splenectomy should continue as first-line therapy for hairy cell leukemia, further study of the safety and efficacy of dCF with longer follow-up will be necessary before dCF can be recommended as first-line therapy outside of the investigational setting.

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CONCISE REPORT

The Percentage of Dense Red Cells Does Not Predict Incidence of Sickle Cell Painful Crisis

By Henny H. Billett, Kenneth Kim, Mary E. Fabry, and Ronald L. Nagel

To test the hypothesis that the tendency of hemoglobin S (HbS) to polymerize within cells is the major determinant of the incidence of vaso-occlusive episodes, we have examined the effect of the percentage of dense cells (as measured by Percoll-Stractan continuous density gradient centrifugation) on the frequency of painful crises in a group of 36 patients with sickle cell disease. No correlation was found between the percentage of dense cells and admissions for crisis. Among the patients with known α-gene status ($n = 25$), the strong correlation between decreased dense cells and α-thalassemia ($-\alpha/\alpha\alpha$) reported previously was confirmed ($P < .001$). In addition, in this small subset, patients with α-thalassemia ($-\alpha/\alpha\alpha$) appeared to have a marginally increased number of admissions for sickle cell crisis ($t = 2.1910$, $P < .05$), which was independent of the percentage of dense cells. We conclude that the percentage of dense sickle cells cannot predict the incidence of painful crisis, suggesting that other factors (microcirculatory regulation or other humoral and cellular factors) are more important in the generation maintenance of painful crises than the necessary, but not sufficient, tendency of HbS-containing red cells to sickle.

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SICKLE CELL DISEASE is a clinically heterogeneous disorder arising from a single amino acid substitution in the hemoglobin (Hb) β-chain, which results in intracellular polymerization of deoxyhemoglobin S.[1] The heterogeneity may express itself by variation in the frequency of painful crises, extent of chronic end organ damage, degree of anemia, or, ultimately, in life expectancy. These varied components of severity may be under different pathophysiological controls, and variation might result from environmental or epistatic gene effects.[2] Sickling has been shown to be modified by cellular HbF content,[3] by the presence of β-thalassemia,[4] and by the mean cell hemoglobin concentration (MCHC).[5] The presence of α-thalassemia has been shown to affect the deformability of homozygous sickle cell anemia (SS) cells and the percentage of dense cells,[6,7] and more recently cation content and transport.[8] In this paper, we will consider the impact of one aspect of the hematologic heterogeneity, the percentage of dense red cells, on the frequency of sickle cell painful crisis. The dense cell fraction contains the largest proportion of irreversibly sickled cells and has been shown to be the most rheologically incompetent.[9] If these qualities of sickle cells are the major determinants of sickle cell painful crisis, as postulated by others,[10] the percent of dense cells should be correlated with the incidence of these episodes. The data presented here do not find that correlation, suggesting that microcirculatory and other physiologic events must be powerful modulators in the incidence of sickle cell painful crisis in patients with sickle cell anemia.

MATERIALS AND METHODS

Patient population. Thirty-six patients with sickle cell disease, followed for a minimum of three years (regardless of their painful crisis record) at the Albert Einstein College of Medicine Heredity Clinic, were chosen for this study. All blood samples were drawn when patients were in a clinical steady state. All patients were homozygous for HbS and 25 were characterized by their α-gene status; 13 of these patients also had concomitant deletional α-thalassemia ($-\alpha/\alpha\alpha$). The number of admissions was calculated from the medical records and confirmed by the patient and the responsible physician in most cases.

Patient characterization. Homozygosity for HbS was determined by two electrophoretic methods and a solubility test. Hematologic characteristics and family pedigree excluded S/β^0-thalassemia in most patients. Hemoglobin levels were determined in EDTA-anticoagulated blood on a Coulter S-plus. HbF levels were determined by agar gel electrophoresis[11] and by alkaline denaturation.[12] α-Gene determinations were performed by Dr Gregory Mears (Columbia Physicians and Surgeons, New York) and Dr Gisela Martinez (Albert Einstein College of Medicine, New York) as previously detailed.[7] Isopycnic red cell continuous density gradients were performed in patients without a history of crisis in the past three weeks, as described by Fabry and Nagel.[13] All statistics were done using a Daisy program on an Apple II computer.

RESULTS

Thirty-six patients (17 males and 19 females) were studied for a mean period ot 58.6 ± 16.5 months. Their average age was 34.6 ± 11.5 years. These patients were characterized by a mean hemoglobin level of 8.52 ± 1.66 g/dL with an average HbF level of 6.35% ± 5.35%. The average rate of admissions for painful crises was 1.64 ± 2.09 per year, and the mean percentage of dense cells was 19.2% ± 13.0%. Average length of stay of 337 hospitalizations was 7.63 ± 7.46 days.

The percentage of dense cells (Fraction 4 [F4]) levels ranged from 1% to 52%. The percentage of the dense cell fraction was not related to hemoglobin level ($r^2 = 0.001$). There was no significant correlation between F4 and the number of admissions for painful crisis ($r^2 = .076$, $F = 2.465$) (Fig 1). Twenty-five of our patients were analyzed for α-gene status; 13 patients had α-thalassemia ($-\alpha/\alpha\alpha$) and 12 patients had four α-genes. As reported previously,[7] there was a marked difference in the F4 levels between patients with three and four α-genes: patients with

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α-thalassemia ($-\alpha/\alpha\alpha$) have a significantly lower dense cell fraction (11.6% ± 7.5% *v* 31.8% ± 11.8%, $t = 5.1538$, $P < .001$). Patients without α-thalassemia ($\alpha\alpha/\alpha\alpha$) showed a strong correlation of F4 with the percentage of HbF ($r^2 = .44$, $F = 7.56$, $P < .03$), but this was not true of those with α-thalassemia. The SS patients with α-thalassemia exhibited an average of three times the number of admissions per year when compared with the non-α-thalassemia group. The difference was marginally significant ($t = 2.1910$, $P < .05$). There was no significant difference in age or hemoglobin level in these two groups, although patients with α-thalassemia appear to have a slightly higher hemoglobin level. These results are listed in Table 1.

The average age of our population is much older than most sample groups of sickle cell patients. When we divided our patients into those 35 and over (n = 12) and those under 35 (n = 20) (the mean age for our group), it is apparent that age is not a factor in crises rate. Patients 35 and over had an average of 1.61 ± 2.60 admissions per year, whereas the younger group had 1.95 ± 2.18 admissions per year. Even patients over age 40 had an admission rate of 1.47 ± 0.82, again not a significant difference from the mean.

DISCUSSION

Only a few systematic attempts have been made to correlate red cell characteristics with painful crisis. Diggs[14] and Rieber et al[15] both reported on the percentage of irreversibly sickled cells during the course of crisis. More recently, Fabry and Nagel[13] have shown that although there is considerable heterogeneity in the percentage of dense cells for each particular patient, the percentage of dense cell fraction was constant (within 10%) during steady state.[16] During episodes of painful crisis, on the other hand, significant decreases in the percentage of dense cells were observed.[16] These findings do not mean that dense cells are indispensable for the generation of crises. We have observed a crisis in a patient who had been depleted of dense cells by a previous crisis,[16] and patients with S/β^0-thalassemia (characteristically exhibiting few dense cells) can also suffer from frequent painful crises. In accord with these observations, the results reported here indicate that there is no correlation between the percentage of dense cells and the incidence of sickle cell painful crises (Fig 1).

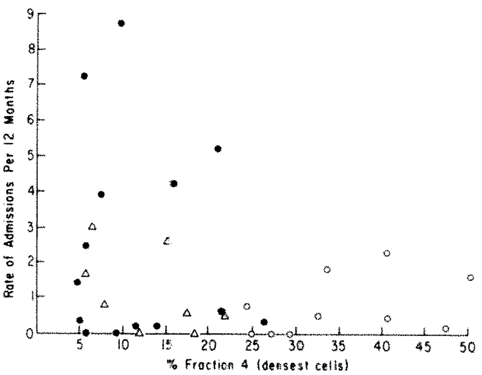

Fig 1. Admissions for painful crisis per year *v* percentage of dense cells (O—$\alpha\alpha/\alpha\alpha$, ●—$\alpha-/\alpha\alpha$, Δ—unknown α-globin gene status).

The percentage of dense cells was found to be strongly associated with ($-\alpha/\alpha\alpha$) thalassemia ($t = 6.805$, $P < 0.001$), in agreement with our previous report[7] and in contrast with the result of Noguchi et al,[17] who find this correlation significant only between ($-\alpha/-\alpha$) and ($\alpha\alpha/\alpha\alpha$) SS patients. Methodological differences in the detection and definition of the dense cell fraction, or differences in the genetic composition of the SS patient population[18] could explain the discrepancy.

Table 1. Effect of α-Gene Status on Several Clinical and Hematologic Parameters in SS Patients

N° α-Genes	3	4
n	13	12
age (yr)	35.69 ± 13.46	35.42 ± 10.69
F4 (%)	11.61 ± 7.48	31.82 ± 11.80*
Adm/yr	2.67 ± 2.96	0.73 ± 0.81†
Hb (g/dL)	9.02 ± 1.40	7.85 ± 2.05
HbF (%)	5.74 ± 5.39	5.23 ± 4.46

Adm/yr, number of hospital admissions for painful crises per year (minimum observation time, three years).

*$P < .001$.

†$P < .05$.

In our sample, SS patients with $-\alpha/\alpha\alpha$-thalassemia had more crisis admissions ($t = 2.191$, $P < 0.05$) than did patients without ($-\alpha/\alpha\alpha$) thalassemia. These marginally significant results are in contrast with published findings of Steinberg and the Cooperative Study of Sickle Cell Disease (CSSCD)[19] in a similar group of SS ($-\alpha/\alpha\alpha$) patients in which no differences were found in incidence of crises among patients with and without α-thalassemia. The ($-\alpha/\alpha\alpha$) sample size in both groups is small (13 in the present study *v* 39 in the CSSCD study). Larger samples defined by α-gene status and probably β-gene cluster haplotype[20] will have to be studied to settle this issue.

When all patients are considered, dense cell fractions appeared to be independent of the percentage of HbF. However, when a correlation was sought between the percentage of HbF and F4 within each of the α-gene groups, a strong correlation emerged between F4 and HbF levels in the non-α-thalassemic SS patient ($\alpha\alpha/\alpha\alpha$). Despite the fact that the HbF levels were virtually identical in both groups (5.74% *v* 5.22%), no correlation of the percentage of HbF and dense cells was seen in ($-\alpha/\alpha\alpha$) concomitant with SS disease, which suggests, as previously noted,[6] that the ameliorating effects of HbF and α-thalassemia are not additive.

The lack of correlation between the percentage of dense cells and the incidence of painful crises, reported here, is an important finding and strongly suggests that a reassessment of the major pathophysiologic determinants of this event is needed. It has been suggested that the propensity to polymerize within the sickle cell should be predictive of painful crises and other elements of severity.[10] This would imply that patients carrying the largest proportion of cells with high MCHC (and hence those most prone to polymerize and exhibit rheological incompetence) should have more frequent painful crises. We interpret our results to mean that, although necessary, the sickle cell is not sufficient to generate a vaso-occlusive episode. We propose that microcirculatory response to initial obstruction (neural or humoral), anatomical characteristics of the vasculature, perfusion pressure regimens, and related events may vary from patient to patient and within each patient from one microcirculatory bed to another. It is the interaction of all these components (some of which are probably under genetic control) that generates the considerable diversity in incidence of painful crises observed among sickle cell anemia patients.

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CONCISE REPORT

Carbonic Anhydrase: A Marker for the Erythroid Phenotype in Acute Nonlymphocytic Leukemia

By Jami Walloch, Stanley Frankel, Mary Ann Hrisinko, and Susan C. Weil

Protein markers are often used to corroborate the morphological subtyping of hematopoietic malignancy. Most commonly, surface markers are used for the phenotyping of hematopoietic cells; however, internal proteins have also been used as markers. Glycophorin, hemoglobin A, hemoglobin F, and transferrin have all been used as markers for the erythroid phenotype. We have recently shown that carbonic anhydrase is constitutively and aberrantly expressed in two erythroleukemic cell lines. We here show that it is also present in high levels in primary erythroleukemic blasts and that it is a useful marker for the M6 phenotype when classifying acute nonlymphocytic leukemia.

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IMMUNOHISTOCHEMISTRY is becoming increasingly more useful in the diagnostic categorization of acute leukemias. Most of the studies have been done on the acute lymphoblastic leukemias, in which a plethora of cell surface, cytoplasmic, and nuclear markers have been used for the characterization of lymphocyte type (B, T, or non-B, non-T cell) and degree of differentiation.[1-4] The acute nonlymphocytic leukemias (M1 to M6, according to the French-American-British [FAB] classification) are less well immunohistochemically characterized; however, a significant body of data involving granulocytic and monocytic markers are accumulating.[5,6] Much less phenotypic data have been gathered on the erythroleukemias (M6). This may be because so few leukemias are erythroleukemias: 95% of acute myeloid leukemias are M1 to M5; only 5% show early erythroid progenitors (M6).

Researchers have recently found erythroid markers useful in identifying early erythroid progenitors. The majority of the studies have been on hemoglobin (both adult and fetal) as a cytoplasmic marker.[7-9] Transferrin has also been used as an erythroid marker[8]; and Andersson et al[10,11] and Greaves et al[12,13] reported that a cell membrane glycoprotein, glycophorin A, is specific for erythroid cells and that glycophorin A appears early in primitive erythroid blasts.

Carbonic anhydrase (CA), the second most abundant protein in red blood cells, has been found at aberrantly high levels in early neoplastic erythroid blasts.[14,15] While three isoenzymes of CA are found in mammalian erythrocytes, the work herein deals exclusively with the most abundant isoenzyme, CAI. In normal human and murine erythroid progenitor cells, a gradual and parallel increase in both CA and globin is observed during maturation. By contrast, in a murine erythroleukemic cell line (Friend or MEL cells) and a new human erythroleukemic cell line (HEL cells),[16] CA is present at high levels in uninduced cells and does not change significantly with induction, whereas a marked increase in globin occurs with chemical induction. In this study, we ask whether this finding in two established continuous erythroleukemic cell lines can be extrapolated to primary human erythroleukemias and if CA may be an early cytoplasmic marker in human neoplastic erythroid cells. Herein, we report eight cases of erythroleukemia (FAB-M6) in which specimens were immunohistochemically stained for adult and fetal hemoglobin and CA.

MATERIALS AND METHODS

The B-5–fixed clot sections or bone core biopsies from eight patients with erythroleukemia (M6) diagnosed by classic morphological criteria[17] were retrieved from cases diagnosed between 1977 and 1985. Also, the B-5–fixed clot sections from five cases each of M1 and M2 acute myelogenous leukemia were obtained. A modified avidin–biotinylated peroxidase complex immunohistochemical staining technique[18] was used in an attempt to classify blast cells with respect to erythroid markers. Rabbit antiadult hemoglobin, antifetal hemoglobin, and anti-CA antibodies were used. The antihemoglobins (adult and fetal) were obtained from Dako (Santa Barbara, Calif); the anti-CA was produced in our laboratory as previously described.[15] Briefly, New Zealand white rabbits were injected subcutaneously with 1 mg of CA-I (Sigma Chemical Co, St Louis) emulsified in an equal volume of Freund's adjuvant. Booster injections were given at 14-day intervals; rabbit serum was collected 14 days after booster injections and assayed by immunoblot analysis with the antigen. Nonimmunized rabbit serum was used as a negative control.

Specimens from each case were stained by immunoperoxidase with all three antibodies, and for each antibody, 500 erythroid cells were evaluated for the degree of positive staining (0 through + + +). Each of these 500 cells was also evaluated for degree of maturity and classified as either early normoblasts or late normoblasts based on the degree of nuclear condensation.

RESULTS

The antihemoglobin antibodies and the anti-CA antibodies did not stain the blasts in the nonerythroid acute nonlymphocytic leukemias, M1 and M2. In these diseases, only rare residual late erythroid cells stained positively.

In the erythroleukemias, many of the cells stained positively. For each antibody, 500 erythroid cells were evaluated and divided into early or late normoblasts. For each of the eight cases, a larger percentage of the erythroid cells stained positively with the anti-CA antibody than with either the antiadult or the antifetal hemoglobin antibody. A typical case is shown in Fig 1. The percentages (average and range) of positively stained cells (in each category) for all eight

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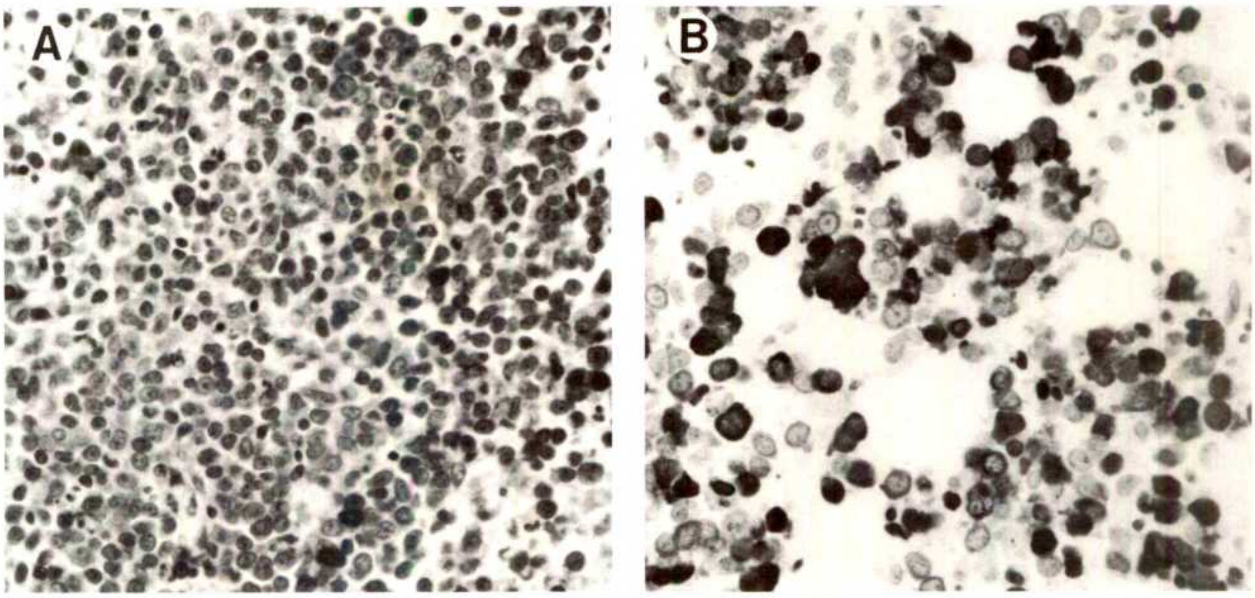

Fig 1. Immunoperoxidase staining with anti-CA on the paraffin-embedded clot section of (a) a case of acute myelogenous leukemia (M2) and (b) one of the eight erythroleukemia (M6) cases described in this study. (Original magnification ×500).

cases are shown in Table 1. The most striking result is that a significantly larger percentage of the early erythroblasts are strongly positive for CA than are positive for either of the hemoglobins (Table 1). This result indicates that the early neoplastic erythroblast has a high concentration of CA.

In the late erythroblasts, the number of cells positive for all three antibodies is similar, but the number intensely positive for CA is markedly higher than the number positive for the hemoglobin (Fig 1). These data suggest that although the production of hemoglobin increases during maturation of erythroblasts, the levels of CA in these neoplastic cells is initially high and remains high.

DISCUSSION

Although no previous work has examined primary erythroleukemic blasts with regard to CA expression, there is an extensive body of data on the CA expression in the peripheral blood cells of various hematologic malignancies. Several cases of juvenile chronic granulocytic leukemia have been described in which the mature red blood cells show a "reversion" to fetal erythropoiesis with high fetal hemoglobin levels and low CA levels.[21] In addition, three cases (polycythemia vera, acute myelogenous leukemia,[7] and erythroleukemia[9]) have been described in which the red cells show high fetal hemoglobin but discordant levels of CA.

Our earlier studies showed that CA is abundantly expressed in the early neoplastic erythroblasts of the erythroleukemia HEL and MEL cell lines in vitro.[15] This result led us to hypothesize that CA may be a more sensitive marker than hemoglobin for neoplastic erythroid cells in vivo. The results of this study (Table 1 and Fig 1) seem to corroborate our hypothesis. Anti-CA does not stain the nonerythroid acute nonlymphocytic leukemias (M1 and M2), but it does stain a significant number of M6 cells positively, especially in the early erythroblasts stage. Anti-CA seems to stain more cells positively in the early stage than does either adult or fetal antihemoglobin, especially if only the intensely staining cells are counted. And although the number of cells staining positively for all three antibodies is similar in the late erythroblast stage, if only the intensely staining cells are counted, anti-CA stains more cells positively than the antihemoglobin antibodies do.

Thus, the aberrant expression of CA has been observed both in vitro and in vivo in human erythroleukemias. The

Table 1. Response of Erythroblasts From Erythroleukemic Patients to Staining With Antihemoglobins and Anti-CA

Antibodies	No. of Early Erythroblasts Average (range)	No. of Late Erythroblasts Average (range)	Positive Early Erythroblasts, + to + + + (%) Average (range)	Positive Early Erythroblasts, + + + Only (%) Average (range)	Positive Late Erythroblasts, + to + + + (%) Average (range)	Positive Late Erythroblasts, + + + Only (%) Average (range)
Antiadult hemoglobin	208	292	33.8	5.7	50.2	9.1
	(128–320)	(180–366)	(10.2–70.4)	(0–27.8)	(35.3–84.0)	(2.7–31.8)
Antifetal hemoglobin	203	297	41.5	6.4	45.1	5.9
	(125–277)	(223–375)	(5.2–73.7)	(0–26.8)	(32.3–75.4)	(0–16.7)
Anti-CA	210	290	65.1	30.6	48.7	26.2
	(128–315)	(185–371)	(34.0–92.4)	(9.8–71.7)	(23.1–66.8)	(10.5–46.5)

appearance of abnormally high levels of CA in erythroleukemia cells may be explained by a change in gene regulation. Debuire et al[19] described extensive sequence homology between the erb A gene of avian erythroblastosis virus and CA genes. It may be speculated that a mammalian oncogene exists that also has sequence homology with CA. An alternative hypothesis suggests that a translocation may juxtapose a CA gene in a manner analogous to the immunogloublin:c-myc (q8;q14) translocation in Burkitt's lymphoma.[20]

A case has been made for the usefulness of glycophorin A as a sensitive and specific marker for early erythroid blasts.[10,13] Greaves et al[12,13] and Andersson et al[10,11] have found glycophorin A particularly useful in reclassifying "cryptic" erythroleukemias that were previously classified as de novo poorly differentiated acute myeloid leukemias (AML) or as acute lymphocytic leukemias (ALL). True AML and ALL seldom express glycophorin A. However, the use of glycophorin A requires fresh patient material, which may not be available for a case that has been misclassified. Thus, CA has the advantage as a marker for cryptic erythroleukemias because it can be used on fixed, paraffin-embedded materials.

Our data indicate that CA is a useful marker for erythroleukemia. For classification of acute nonlymphocytic leukemias, it is specific for M6 and more sensitive than either adult or fetal hemoglobin. An added advantage of CA as a marker is that it can be used on fixed, paraffin-embedded file material. CA may thus be useful in confirming erythroid phenotype and reclassifying poorly differentiated AML and ALL as M6.

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CONCISE REPORT

Platelet Membrane Microparticles in Blood Bank Fresh Frozen Plasma and Cryoprecipitate

By James N. George, Elaine B. Pickett, and Robert Heinz

Cryoprecipitate has been demonstrated to correct the bleeding abnormality of patients with some congenital (storage pool disease) and acquired (uremia) platelet abnormalities, but the reason for this effect is unknown. We found significant platelet contamination in plasma harvested to prepare fresh frozen plasma and cryoprecipitate. The platelet membrane microparticles produced by freezing and thawing of the plasma were highly concentrated in cryoprecipitate and may contribute to its therapeutic effect.

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THE CRYOPRECIPITATE FRACTION of plasma can correct the bleeding abnormality of some patients with disorders of platelet function. The original observation was made in 1978, by Gerritsen et al,[1] who studied eight patients with congenital platelet storage pool deficiency. In each patient, the infusion of 16 bags of cryoprecipitate caused a shortening of the bleeding time, and four of these patients had surgical procedures during cryoprecipitate treatment with no excessive bleeding.[1] Infusion of pasteurized human albumin into six of the patients had no effect on the bleeding time.[1] This observation was extended by Janson et al[2] to the treatment of seven uremic patients. The bleeding time was decreased to normal, or nearly normal, in each patient after the infusion of ten bags of cryoprecipitate, and four of the patients underwent major surgery with no bleeding complications.[2] However, there was no control group in this study, and the risks of bleeding with surgery are unknown.

Although the explanation for this effect of cryoprecipitate is unknown, its use is commonly suggested in current textbooks.[3-7] The active principal in cryoprecipitate is believed to be a plasma component, and a potential advantage mentioned for its use in these patients is that platelet alloimmunization can be avoided.[1,7] However, the appearance of antiplatelet antibodies has been observed in patients with von Willebrand's disease treated only with cryoprecipitate (S.L. Pfueller, Monash University, Victoria, Australia, personal communication, October 1985), as well as in animals immunized with factor VIII–von Willebrand factor prepared from cryoprecipitate.[8] To determine if platelet-derived material is present in blood bank plasma products, we measured the platelet concentration in fresh plasma and the resulting platelet membrane microparticles[9,10] after the preparation of fresh frozen plasma and cryoprecipitate.

MATERIALS AND METHODS

Plasma samples were obtained from routine normal donors at the Medical Center Hospital Blood Bank, and plasma fractions were prepared by the Blood Bank personnel using standard methods described in the technical manual of the American Association of Blood Banks.[11] An average of 465 mL of blood in our experiments was collected into 63 mL of CPDA-1 anticoagulant in Fenwall PL-146 triple-unit bags. PL-732 bags were used for the platelet concentrates. When platelet concentrates were being made, the blood was centrifuged at 1,800 g for three minutes at room temperature in a RC3-B centrifuge with a horizontal H-6000A rotor (Sorvall Instruments, Wilmington, Del), and the supernatant platelet-rich plasma (PRP) was squeezed off by a plasma expressor (Fenwall Laboratories, Deerfield, Ill). The PRP was then centrifuged at 5,000 g for five minutes at room temperature and the supernatant plasma was expressed off: an average of 157 mL of plasma was removed and 53 mL of plasma left with the platelet concentrate. The supernatant plasma was promptly frozen at −65 °C. Alternatively, the whole blood was centrifuged at 5,000 g for five minutes at room temperature and the plasma was expressed off and promptly frozen. An average of 244 mL of plasma was removed with five donors; therefore, about 70 mL of plasma plus anticoagulant were left with the cells. Samples for platelet counts (Thrombocounter, Coulter, Hialeah, Fla) were obtained from the plasma bags before freezing. Because of the greater volume, cryoprecipitate is prepared in our blood bank only from the plasma that had been separated directly from whole blood. The plasma was frozen at −65 °C and then placed at 4 °C to thaw overnight. It was centrifuged at 5,000 g for eight minutes at 4 °C and the supernatant "cryo-poor plasma" expressed off, leaving an average of 13 mL of plasma with the precipitate to refreeze at −65 °C. Whole blood samples were obtained directly from the phlebotomy tubing after the completion of the donation, anticoagulated with 5 mmol/L EDTA, 1 μg/mL prostaglandin E_1, and 0.1 mol/L dibutyryl cyclic adenosine monophosphate, and centrifuged at 12,000 g for ten minutes to obtain plasma for the assay of platelet membrane microparticles.[10]

Platelet membrane microparticles were measured as previously described,[10] using the radioiodinated mouse monoclonal antibody, Tab,[12,13] against glycoprotein (GP) IIb. Plasma samples (0.5 mL) were incubated with 0.5 μg of ^{125}I-Tab for 30 minutes at room temperature. Control samples, subtracted from the plasma samples, contained 0.5 μg of ^{125}I-Tab in buffer. After incubation, the samples were diluted to 7.2 mL with Tyrode's buffer containing 5 mmol/L EDTA and 0.35% bovine serum albumin and centrifuged at 67,000 g at 4 °C for 60 minutes. The buffer was aspirated and the invisible pellet was washed once with 7.2 mL of buffer at 67,000 g for 20 minutes. Then the cpm remaining in the pellet were measured and expressed as molecules of GP IIb per milliliter of plasma, assuming

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that one molecule of Tab binds to one molecule of GP IIb. Because the membrane glycoprotein composition of platelet microparticles in plasma is similar to platelets,[10] the measurement of a single membrane glycoprotein, GP IIb, should provide a reasonable estimate of plasma microparticle concentration.

RESULTS

Significant numbers of platelets were present in the plasma used for fresh frozen plasma and to prepare cryoprecipitate. Plasma prepared from PRP contained more platelets than plasma prepared by a single centrifugation of whole blood (33,420 ± 5,615 (SD, n = 5) *v* 13,796 ± 5,204 (n = 5) platelets per microliter, $P < .001$). The centrifugation force is the same for both preparations and sufficient to sediment all platelets.[9] More platelets may be present in the plasma separated from the platelet concentrates because less plasma is left with the platelet pellet than is left above the platelet–white cell layer when the whole blood is separated by the single fast centrifugation. Also, the presence of red cells may affect platelet sedimentation. After frozen storage, as for clinical use, intact platelets were lysed and platelet membrane microparticles were present in the plasma proportional to the original platelet content (Fig 1). The correlation was significant ($r = .89$, $P < .001$). Therefore, the microparticle concentration in fresh frozen plasma prepared from PRP was greater than in fresh frozen plasma prepared directly from whole blood (Table 1). The microparticle concentrations of the donors' original plasmas, obtained by a method to prevent any platelet contamination or activation, were all normal (normal values = 1.95 ± 1.37 (SD) × 10^{10} molecules of GP IIb per milliliter plasma, n = 21, ref 10) and therefore were a negligible contribution to these blood bank plasma samples. The platelet membrane microparticles were significantly concentrated within the cryoprecipitate. In these five samples, the microparticle concentration of cryoprecipitate was 29-fold that of the cryosupernatant plasma, with a range of 20- to 34-fold. The cryoprecipitate contained only 5% of the volume of the original plasma but 48% of the total microparticles. The microparticle concentration was 265-fold that of the donors' original plasma. Using an assumption that the platelet content of GP IIb equals 72,358 molecules per cell (the value we measured by ^{125}I-Tab binding to intact platelets after thrombin-induced secretion, assuming that platelets have no intracellular pool of GP IIb[11]), ten bags of cryoprecipitate would contain an amount of platelet membrane GP IIb equivalent to about 4 × 10^9 platelets, or about 20 mL of blood.

Table 1. Platelet Membrane Microparticles in Fresh Frozen Plasma and the Cryoprecipitate and Cryosupernatant Fractions of Plasma

Preparation	Platelet Microparticles (Molecules GP IIb per mL × 10^{-10})	Volume (mL)
Original donor plasma	0.9 ± 0.5	
Fresh frozen plasma prepared from PRP	100 ± 47	157
Fresh frozen plasma prepared from whole blood	25 ± 5	244
A. Cryoprecipitate	228 ± 30	13
B. Cryosupernate (cryo-poor plasma)	8 ± 2	224

The data are the mean values ± 1 SD for ten samples of cell-free plasma obtained directly from the donors, 13 samples from fresh frozen plasma bags obtained from platelet-rich plasma (PRP) during the preparation of platelet concentrates, and five samples from fresh frozen plasma bags prepared directly from whole blood. The concentration of platelet membrane microparticles was greater in the plasma prepared from PRP than in plasma prepared from whole blood ($P < .005$). The five plasma bags prepared from whole blood were subsequently fractionated into cryoprecipitate and the supernatant "cryo-poor plasma" components, with a significant concentration of platelet membrane microparticles in the cryoprecipitate fraction compared with the whole plasma ($P < .001$). Platelet membrane microparticles were measured using ^{125}I-Tab, a mouse monoclonal antibody to glycoprotein (GP) IIb.

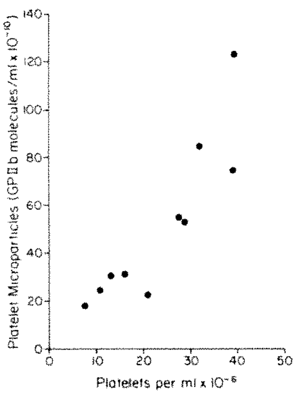

Fig 1. Platelet membrane microparticles in fresh frozen plasma. The number of residual platelets in the fresh plasma immediately after separation from whole blood or from PRP was proportional to the concentration of membrane microparticles present after freezing and thawing (correlation coefficient, $r = .89$, $P < .001$). The data are from five bags obtained from centrifugation of whole blood and five bags obtained from centrifugation of PRP during the preparation of platelet concentrates.

DISCUSSION

Plasma separated to prepare the standard blood bank products, fresh frozen plasma and cryoprecipitate, contained a significant number of platelets. When platelet concentrates were prepared, the resulting supernatant plasma contained more platelets than if the plasma was harvested directly from the whole blood. Platelet counts as high as 39,000 per microliter were observed. Platelet contamination of bank plasma products may have clinically relevant effects, such as cleavage of von Willebrand factor by the platelet calcium-activated protease with loss of the largest multimers.[14] Proteolytic alteration of fibrinogen and fibronectin due to platelet protease activity may also occur.[15,16]

The concentration of platelet membrane microparticles in fresh frozen plasma after thawing was proportional to the number of platelets originally present, and these microparticles were highly concentrated by cryoprecipitation. This process concentrates the large contact-promoting proteins that interact with the platelet surface,[17] fibrinogen, fibronectin, and von Willebrand factor, as well as factor VIII and other proteins,[16,18] and platelet membrane microparticles may be concentrated by an interaction with these proteins. We have found that platelet microparticles in plasma contain the membrane GP Ib and GP IIb–IIIa that can interact with these proteins.[10] The ability of platelet microparticles to bind to fibrinogen, fibronectin, or von Willebrand factor may be enhanced by cryoprecipitation, similar to the enhanced ability of platelets to interact with fibrinogen and von Willebrand factor that are bound onto a solid support.[19-21] Red cell fragments may also be concentrated in cryoprecipitate, as the cryoprecipitate from $Rh(D)^+$ donors can immunize $Rh(D)^-$ patients.[22]

A hemostatic function for platelet microparticles remains speculative. The amount of microparticles in ten to 16 bags of cryoprecipitate[1,2] is only equivalent to the platelets in about 20 to 30 mL of blood, but the dispersion of the platelets' contact-promoting membrane glycoproteins into small particles may amplify their effectiveness. An analogous phenomenon is the shedding into media by tissue culture cells of membrane microparticles, termed adherons, that promote both cell adhesion to the substratum and cell–cell aggregation.[23] This function can be blocked by a monoclonal antibody to a 170,000-molecular weight membrane protein shared by the intact cells and adherons.[24] The mechanism of adheron function is unknown, but it is assumed to relate to the concentration of cell adhesion molecules in small particles that can facilitate contact between cells and surfaces.[23] A similar property may be postulated for platelet membrane microparticles.

Because our data were obtained by standard procedures[11] in a general hospital blood bank, these observations should be relevant to plasma components prepared for routine clinical use. Therefore, the presence of platelet membrane microparticles must be considered in interpreting the effects of transfused plasma products.

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CONCISE REPORT

Spurious Detection of Terminal Deoxynucleotidyl Transferase in Phytohemagglutinin-Stimulated Lymphocytes

By J.A. Fletcher, R. Bell, M. Koekebakker, A.A. Dowers, R.P. McCaffrey, and R.D. Barr

Expression of terminal transferase (TdT) is believed to be restricted to primitive lymphoid cells; recently, however, indirect immunofluorescent (IF) assays have been used to demonstrate the apparent presence of TdT in phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes and in various nonlymphoid malignancies. Using an IF assay, we found that a heteroantiserum to TdT reacted with cultured and PHA-stimulated human peripheral blood mononuclear cells, but we were unable to confirm the presence of TdT in these cells using immunoblotting and biochemical assays. We conclude that the IF results are spurious and most likely represent recognition by the heteroantiserum of inducible protein(s) other than TdT.
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TERMINAL deoxynucleotidyl transferase (TdT) is a unique template-independent DNA polymerase that is expressed, in the normal host, only in primitive T and B lymphoid cells. The biochemical properties and limited anatomic expression of TdT suggest that the enzyme may be responsible for genomic alterations which contribute to the generation of somatic immunologic diversity,[1] but this cellular role remains speculative. Nevertheless, TdT assays have become clinically important over the past decade; elevated levels of TdT in acute leukemia, non-Hodgkin's lymphoma, and CML-blast crisis specimens, indicate that the disease is biochemically lymphoblastic in nature and that there is a high likelihood of response to conventional vincristine-prednisone-based chemotherapy regimens.[2,3]

The primitive T and B lymphocytes, which normally express TdT, are restricted to the thymic and bone marrow compartments; there are very few circulating TdT-positive cells.[4,5] However, several recent reports describe the identification of TdT-positive cells, by indirect immunofluorescent (IF) methods, in peripheral blood mononuclear cells following viral stimulation,[6] in patients with AIDS,[7] culture in a diffusion chamber,[8] and phytohemagglutinin (PHA) stimulation.[9] Because biochemical assays have not revealed enzymatic TdT activity in PHA-stimulated human mononuclear cells,[10,11] it may be that the IF assays in these instances have detected a TdT-like protein that is biologically inactive ("true-positive" result) or a non-TdT protein that cross-reacts with components of some anti-TdT reagents ("false-positive" result). To characterize the questionable presence of TdT in cultured and PHA-stimulated human mononuclear leukocytes better, we studied these cells with an immunoblot detection system that is sensitive and specific for TdT.

MATERIALS AND METHODS

Cells. Mononuclear cells, obtained from a healthy volunteer (subject A), were isolated by density-gradient separation of 200 mL of heparinized blood on Ficoll-Hypaque. These cells were analyzed for TdT using the IF and immunoblot assays at the time of cell separation (day 0) and after 5 days of culture in the presence (day 5 + PHA) and absence (day 5 − PHA) of PHA. Mononuclear cell preparations were obtained, in identical fashion, from four other healthy volunteers (subjects B, C, D, and E) manipulated as above, and then pooled to allow sufficient cell numbers for biochemical assays of TdT activity; these samples were also studied with the immunoblot assay. Leukemic cells and calf thymocytes were harvested and processed as described previously.[12]

Immunofluorescent assay. IF assay was performed as described previously.[13] Unfixed cytocentrifuge preparations ($\sim 5 \times 10^5$ cells per slide) were incubated sequentially with rabbit anti-calf TdT serum (P.L. Biochemicals, Milwaukee) and FITC-IgG goat anti-rabbit IgG (Cappel Laboratories, Cochranville, Penn). Results were expressed as percentage of total cells showing a granular pattern of fluorescence (Fig 1A).

Biochemical assay. The biochemical assays were performed as described previously[14] using phosphocellulose column fractions prepared from the lysates of 0.4 to 1×10^8 cells. The reaction mixtures contained ^{3}HdGTP and oligo(dA)$_{50}$, and calf thymocytes served as the positive control.

Immunoblot Assay. Cells (1×10^7) were lysed by sonication at 4 °C in 0.5 mL of RIPA buffer (0.02 mol/L of Tris, pH 7.5; 0.1 mol/L of NaCl; 200 KIU/mL of Aprotinin; 1 mmol/L of EDTA; 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) (Sigma, St Louis); 0.5% Triton-X). Lysate protein concentrations were determined using the BioRad protein assay (BioRad, Richmond, Calif), and 40 µg of each lysate was dissolved in Laemmli sample buffer and subjected to Western blotting following standard procedures.[15] The western blots were incubated sequentially with: (a) 6% goat serum; 0.05% Tween-Tris-buffered saline at 30 °C for 30 minutes; (b) 1:60 dilution of partially purified rabbit anti-calf TdT (P.L. Biochemicals) in 2% goat serum; 0.05% Tween-Tris-buffered saline at 4 °C for 24 hours; and (c) 1:1,000 dilution of peroxidase linked goat anti-rabbit IgG (Cappel Laboratories) at 30 °C for 1 hour. The peroxidase activity was then visualized using 4-chloro-1-naphthol and H_2O_2.

RESULTS

The immunoblot protocol was carried out initially on a series of positive controls that included human thymocytes and nine enzymatically TdT-"positive" acute leukemia samples; the negative controls were five acute leukemia samples that were known to lack enzymatic TdT activity. The positive controls uniformly showed a band of a mol wt of ~60 kd, which is the weight of nonproteolyzed human TdT,[16,17]

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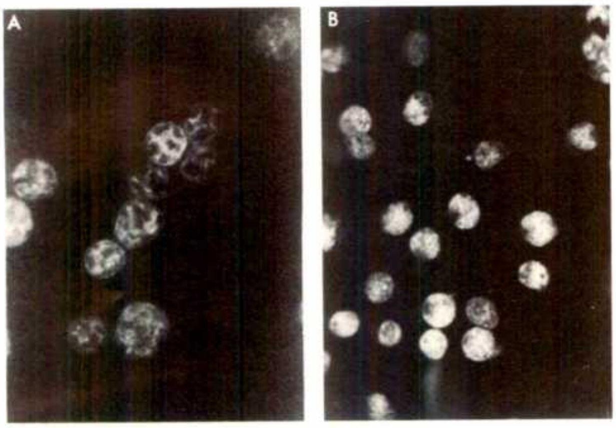

Fig 1. Immunofluorescent staining for TdT. (A) Acute lymphoblastic leukemia cells (biochemically TdT$^+$). (B) Peripheral blood mononuclear cells that have been cultured for 5 days in the presence of PHA.

whereas the negative controls showed no such band (Fig 2A). In mixing studies of the TdT-positive and TdT-negative specimens shown in Fig 2A, the TdT immunoblot assay was able to detect a 1% population of TdT-positive cells (data not shown).

The peripheral blood mononuclear cells obtained from subject A were negative for TdT by immunofluorescence at day 0; after 5 days of culture with and without PHA, however, these cells were, respectively, 72% and 61% TdT-positive by IF (Fig 1B); none of these samples was found to have TdT by the immunoblotting protocol (Fig 2B). The parallel experiment, using pooled mononuclear cells from subjects B, C, D, and E, revealed no elevations (all samples <0.2 U/10^8 cells) of enzymatic TdT activity at day 0, day 5 + PHA, and day 5 − PHA; again, each of these samples was negative for TdT by the immunoblotting method.

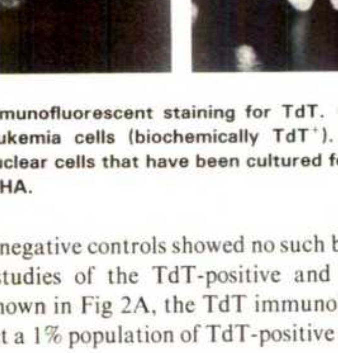

Fig 2. (A) TdT immunoblot of chronic myelogenous leukemia in blast cell crisis (lane 1) and acute myelogenous leukemia (lane 2). Mol wt markers are indicated in kilodaltons. Lane 1 shows a single band at 60 kd, which is the known mol wt of nonproteolyzed human TdT. The CML-BC sample had enzymatic TdT activity, whereas the AML sample lacked enzymatic TdT activity. (B) TdT immunoblots of peripheral blood mononuclear cells at day 0 (lane 1), after 5 days of culture in the absence of PHA (lane 2), and after 5 days of culture in the presence of PHA (lane 3). Also included is an acute lymphoblastic leukemia sample (lane 4) that was known to have enzymatic TdT activity. The ALL sample has a strong band at 60 kd that is not present in any of the peripheral blood mononuclear cell groups. The day 5 + PHA cells show a strong band at ~75 kd that is also faintly present in the day 5 − PHA and ALL cells.

DISCUSSION

In several previous investigations, IF methods have been used to document TdT activity in cultured and PHA-stimulated adult peripheral blood mononuclear cells,[8,9] suggesting that blastic transformation of lymphocytes may be accompanied by TdT expression. Cyclic AMP has been shown, by immunoprecipitation and biochemical assays, to induce TdT expression in lymphocytes, but this induction is restricted to pre-B cells.[18] We have confirmed that cultured and PHA-stimulated adult peripheral blood mononuclear cells show "TdT activity" when examined by an established IF assay; however, these same cells do not show TdT activity when examined by biochemical and immunoblotting techniques.

Although our IF results demonstrate that certain anti-TdT reagents react with cultured and mitogen-stimulated lymphocytes, the absence of enzymatic TdT activity in these cells and the absence of the characteristic 60-kd human TdT band with immunoblotting lead us to believe that the anti-TdT reagent is reacting with inducible proteins other than TdT. These findings also suggest that previous immunofluorescent and immunohistochemical determinations of apparently strong TdT activity in nonmalignant bone marrow and peripheral cells[19,20] and in nonlymphoid malignancy[21] may represent a similar recognition of inducible non-TdT proteins by heterospecific anti-TdT reagents. We were not able to discriminate between reactivity with these non-TdT proteins and with actual TdT based on the morphologic pattern of IF staining.

Several of the day 5 − PHA and day 5 + PHA immunoblots revealed a strong band at a mol wt of ~75 kd; this may represent the protein that is recognized in the IF assays. There are several known T cell antigens in this mol wt region, but the number of proteins synthesized by mitogen-activated lymphocytes is large,[22] and the actual identity of this 75-kd entity is unknown.

The poor specificity of the TdT antiserum used for our IF assays should be partially rectified with the use of affinity-purified reagents. Unfortunately, the available affinity-purified reagents have shown suboptimal sensitivity in IF assays,[13] and it remains to be seen whether monoclonal antibodies to TdT[23,24] will allow increased specificity in IF and immunohistochemical testing without significant loss of sensitivity. In general, either the biochemical or IF TdT assay can appropriately be used to classify acute hematologic malignancies, as both assays have shown good concordance when used to diagnose acute leukemias and lymphomas of the lymphoblastic type.[25] The IF assay, however, has been unreliable in detecting relapse of leukemia or lymphoma.[6,9] Therefore, positive IF results in this context should be confirmed by the immunoblot or other independent assays; this same recommendation applies to the IF detection of TdT in nonhematologic malignancies and in viral or mitogen-stimulated lymphocytes. The finding of TdT induction, when documented by IF alone, should be interpreted with caution.

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CONCISE REPORT

Effects of Recombinant GM-CSF on the Blast Cells of Acute Myeloblastic Leukemia

By T. Hoang, N. Nara, G. Wong, S. Clark, M.D. Minden, and E.A. McCulloch

The effects of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) were compared to those of media conditioned by the continuous bladder carcinoma line, HTB9 (HTB9-CM), using three criteria. First, both GM-CSF and HTB9-CM stimulated blast colony formation in methylcellulose cultures, patient-to-patient variations were seen in the dose–response curves, and GM-CSF was effective, but less so than HTB9-CM. Second, GM-CSF also enhanced growth of blast progenitors in suspension culture, indicating its capacity to support self-renewal. GM-CSF was as effective as HTB9-CM in the production of adherent cells during the growth of blast cells in suspension, a finding that is interpreted to mean that GM-CSF also supports postdeterministic events in blast differentiation. Finally, colonies growing in the presence of GM-CSF were not phenotypically different than those stimulated by HTB9-CM.

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A MOLECULAR clone has been isolated for the hematopoietic growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF)[1]; the factor has been transfected into Chinese hamster ovary (CHO) cells and a line that continuously produces GM-CSF has been obtained.[2] The availability of this recombinant GM-CSF makes it possible to test its effects on various target cells.

GM-CSF was first described as a growth requirement for granulopoietic-macrophage colony formation in culture.[3] It also supported the initial divisions of multipotential and erythropoietic precursor cells, but further differentiation and proliferation of these cells required other growth factors.[4] With recombinant GM-CSF at high concentrations, the requirement for additional stimulator was no longer seen.[5] In this article, we report the effects of recombinant GM-CSF on blast cells from patients with acute myeloblastic leukemia (AML). We find that GM-CSF stimulates both the formation of blast colonies in methylcellulose[6] and the growth of blast progenitors in suspension.[7] In addition, recombinant GM-CSF increases the production of adherent AML blasts, cells that have been shown previously to have some of the phenotypic characteristics of macrophages.[8]

MATERIALS AND METHODS

Cells

With the patients' informed consent, cells were obtained from the peripheral blood of three patients with AML. The sample from patient 1 (French-American-British classification, FAB 4) was obtained at relapse following treatment with high-dose cytosine arabinoside.[9] Patient 2 (FAB 1) was seen at first occurrence and was treated with a combination of Adriamycin, cytosine arabinoside, and thioguanine.[10] Patient 3 (FAB 4) was in relapse following treatment with high-dose cytosine arabinoside.[9]

The suspensions were depleted of T cells by sheep erythrocyte rosetting as described previously.[11] Normal bone marrow cells were obtained from marrow transplant donors; these populations were depleted of adherent cells by overnight incubation in Iscove's medium (GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS, GIBCO). The human bladder carcinoma cell line (HTB9) was obtained from Dr Robert Phillips of this institute; the cells are maintained in α-medium (α-MEM) (GIBCO) supplemented with 4% FCS.

Source of Growth Factors

Conditioned medium from HTB9 and recombinant GM-CSF. HTB9-CM was harvested on day 7, 2 to 3 days after the cells became confluent. The HTB9-CM was sterilized by filtration (Millipore, .22 μ-m) and stored at 4 °C. One batch, selected for its potency as a stimulator of colony formation by AML blasts, was used without purification as a standard in all experiments. Recombinant GM-CSF was obtained from the Genetics Institute, Boston, as unpurified supernatant from GM-CSF-transfected CHO cells. This material contains 1% bovine serum and $>10^5$ U of GM-CSF.

Cell cultures. Recombinant GM-CSF was compared with HTB9-CM in cultures of blast cells from patients with AML and marrow cells from normal donors. The effect of growth factors on blast colony formation was tested by plating cells in 96-microwell plates (Linbro, Flow Lab, McLean, Va) as described previously, in α-MEM supplemented by 10% FCS and 0.8% methylcellulose.[12] Each well contained from 10^3 to 10^4 cells in 100 μL of medium; these cell concentrations were selected to yield a minimum number of colonies in the absence of added factor. The putative stimulators to be tested were added to the wells in dilutions ranging from 10^{-7} to 10^{-2}. The cultures were incubated for 5 to 7 days in a moist atmosphere containing 5% CO_2, and colonies were enumerated using an inverted microscope.

Blast cells were cultured in suspension in 35-mm Petri dishes (Lux) in α-MEM and 20% FCS as described previously.[7] Cells from patient 1 can be maintained for long periods in suspension; their exponential growth for 224 days has been described[13] and still continues; growth remains dependent on the addition of HTB9-CM to the cultures. Growth factors were added at the same range of dilution given above. After 7 days, the cells were harvested and plated in methylcellulose with an optimal concentration of HTB9-

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CM (10%). The recovery of clonogenic cells was obtained by multiplying the plating efficiency in methylcellulose by the number of cells harvested from the suspension.

The assay for adherent cells was a minor modification of that described by Langley and colleagues[8] in which blast cells were cultured in suspension in Lux tissue culture microwells for 7 days. The nonadherent cells were then removed, and the residual adherent cells were counted using an inverted microscope. Based on cell sources, the kinetics of development of the adherence property, and the cell phenotypes, we concluded that the adherent cells belonged to the blast population.[8]

Normal nonadherent bone marrow cells were plated at a concentration of 5 × 10^4 in 35-mm Petri dishes in Iscove's medium supplemented with 10% FCS, deionized bovine serum albumin (BSA) (Hoechst, Calbiochem-Behring, La Jolla, Calif, electrophoretically pure, 10 mg/mL), iron-saturated human transferrin (Hoechst, 360 mg/mL), α-thioglycerol (10^{-4}mol/L), purified homogeneous erythropoietin (obtained from the Terry Fox Laboratory, University of British Columbia, 1 U/mL), and methylcellulose. As in the previous assays, various concentrations of growth factors were added; colonies were assessed both for number and morphology with an inverted microscope after 7 and 14 days of incubation. The concentration required for half-maximal stimulation of granulocyte and macrophage colonies was defined as 1 U of activity.[12] The batch of recombinant GM-CSF used for the present study contained 1.2 × 10^5 U/mL. At plateau concentration, it stimulated the same number of granulocyte and macrophage colonies as HTB9-CM. In the presence of erythropoietin, it also supported the growth of multipotential and large erythroid colonies.

Monoclonal antibodies. Phenotypes of blast cells were determined before and after culture, using indirect immunofluorescence, as described previously.[14] The antibodies used were My-1,[15] My-7, and My-9[16,17] that recognize granulopoiesis-associated antigens, and MO1[18] that binds markers of both granulopoietic cells and macrophages.

RESULTS

Titrations

Titration of GM-CSF on blast colony formation in methylcellulose, growth of clonogenic blast progenitors in suspension, and the generation of adherent blast cells are shown in Fig 1. Panel A depicts the increase in blast colonies observed with increasing concentration of GM-CSF. Data from three patients are shown. For patient 1, the cells were obtained after frozen cells had been maintained in suspension culture for 3 weeks. Cells from the other patients were tested when obtained or from cyropreserved samples. Patient-to-patient variation is evident. However, in each instance, the dose–response curve extended over several tenfold dilution steps. The maximum values for colony formation obtained with a standard HTB9-CM preparation are given in the legend to Fig 1. In no instance was this value obtained with GM-CSF, although no obvious differences in colony size were noted. Indeed, for patient 1, only 19% of the colonies stimulated by HTB9-CM were seen with a concentration of GM-CSF much greater than that required for maximum stimulation of granulopoietic colonies (8,000 U/mL).

Panel B shows the effects of increasing GM-CSF concentration on recovery of clonogenic blasts after 7 days in suspension culture. Again, a dose-related increase was seen, although the form of the dose–response curve was different for the two samples depicted in panel B of Fig 1. Cells from patient 1 were tested after almost 1 year in continuous suspension culture; their response to recombinant GM-CSF was similar to that seen with HTB9-CM (see legend to Fig 1). For patient 2, GM-CSF was less effective than HTB9-CM.

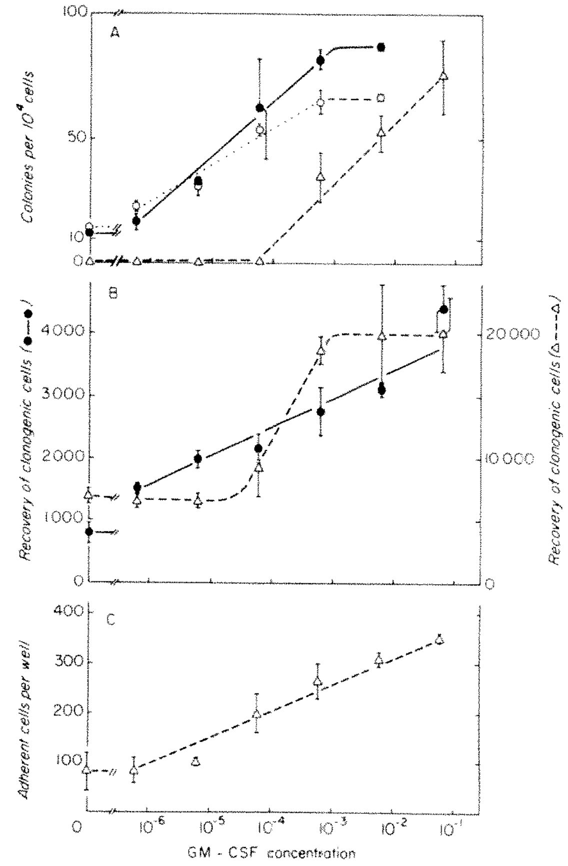

Fig 1. Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) dose–response curve. (A) In methylcellulose cultures of blast progenitors. Maximum stimulation by HTB9-CM was: 430 ± 20 (AML 1, Δ---Δ), 155 ± 7 (AML 2, ●—●), 148 ± 6 (AML 3, O---O). (B) In suspension cultures. Recovery in clonogenic cells with HTB9-CM was: 47,000 ± 3,400 (AML 1) and 3,860 ± 290 (AML 2). Symbols as in A. (C) In the generation of adherent cells. The input cell number was 10^4 per well. Maximum number of adherent cells per well with HTB9-CM was: 405 ± 27 (AML 1). Symbols as in A.

The generation of adherent blast cells in suspension cultures is shown in panel C as a function of GM-CSF concentration. The cells were from patient 1, tested after many months in suspension culture. Again a dose response was obtained, reaching almost the same value as that seen with HTB9-CM, even though the cells were those that responded poorly to GM-CSF in the assays based on proliferation (patient 1). The same increase in adherent cells in the presence of GM-CSF was seen with samples 2 and 3 (data not shown).

Phenotypes

Using monoclonal antibodies directed against granulopoietic and monocytic differentiation antigens, the phenotypes of blast cells were determined before plating and from blast colonies. Results of an experiment representative of four experiments are given in Table 1. In agreement with past findings,[19] myelopoietic markers increased during colony formation. Evidence of granulopoietic maturation was not seen. No difference was observed between colonies grown in the presence of GM-CSF as compared with those grown in the presence of HTB9-CM.

Table 1. Surface Markers on Colony Cells

	MO1 (%)	My-1 (%)	My-7 (%)	My-9 (%)
Before culture	25	12	23	74
After culture				
Control	30	44	79	63
GM-CSF	15	54	36	78
HTB9-CM	37	76	45	30

Results expressed as percentage of positive cells (AML 1).

DISCUSSION

The major finding of this article is that recombinant GM-CSF is able to stimulate blast colony formation in methylcellulose cultures[6] and the growth of blast progenitors in suspension. The latter assay is considered to reflect the renewal potential of blast stem cells;[7] furthermore, colonies grown in methylcellulose cultures in the presence of recombinant GM-CSF were replated successfully (data not shown), confirming the ability of GM-CSF to support self-renewal.[20] Finally, the capacity of GM-CSF to increase the number of adherent cells is evidence for its support for differentiation-like events, since adherent blast cells usually show increased expression markers often associated with macrophages as well as those characteristic of the blast population.[8]

Recently, we reported[13] that supernatants from cultures of the continuous cell line HTB9 (HTB9-CM) were effective stimulators of blast colony formation. On the basis of partial separation of Sephadex G100, we suggested that the active growth factor for blast colony formation (leukemic blast growth factor or LBGF) might not be identical to GM-CSF. The present study does not contradict that suggestion, since GM-CSF was usually a much less powerful stimulator than HTB9-CM. Rather, the data is now consistent with the possibility that colony formation or growth of blast progenitors may require more than one factor, working singly or in combination.

Our data confirm the findings of others that GM-CSF will stimulate multilineage colonies as well as colonies containing only granulocytes and macrophages,[4,5] a finding that prevents the use of GM-CSF response for lineage definition. In the context of this report, the finding that GM-CSF is active on AML blast progenitors is not evidence that these cells belong exclusively to a lineage related to granulopoietic or macrophage differentiation. In the experiment reported here, it is unlikely that adherent cells not from the leukemic clone were detected, since the blasts had been maintained for >1 year with repeated subcultures.

Finally, our results may have practical significance. GM-CSF has been considered as a putative therapeutic agent. Indeed, preclinical trials have been reported.[21] The ability of GM-CSF to stimulate the growth of blast progenitors suggests that it may be contraindicated in the treatment of any condition related to AML or capable of progression to that disease.

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CONCISE REPORT

Fragment D-Dimer Levels: An Objective Marker of Vaso-occlusive Crisis and Other Complications of Sickle Cell Disease

By Dana V. Devine, Thomas R. Kinney, Patricia F. Thomas, Wendell F. Rosse and Charles S. Greenberg

Although abnormalities in coagulation tests have been reported during vaso-occlusive crises in patients with sickle cell disease, objective, readily performed laboratory tests that document the occurrence of this complication have not been available. We examined the relationship between fibrin D-dimer levels and the occurrence of complications in patients with sickle cell disease, using a commercially available latex bead agglutination assay. The patients were either asymptomatic, hospitalized for vaso-occlusive crisis, or had other complications of sickle cell disease including leg ulcers, chronic cholecystitis, aseptic necrosis, joint pain and infection. Fifty-seven percent of 187 samples on 96 patients had elevated levels of fibrin D-dimer. Ninety percent of 75 samples from asymptomatic patients were negative for fibrin D-dimer (<1 μg/ml) but 97% of 29 samples from patients with vaso-occlusive crisis and 85% of 83 samples from patients with other complications of sickle cell disease were positive. In serial studies, worsening or amelioration in clinical complications were reflected in increasing or decreasing levels of fibrin D-dimer, respectively. The molecular species of fibrin identified by the latex agglutination test was shown to be fragment D-dimer by successive immunoprecipitation and protein blot analysis. We conclude that the complications of sickle cell disease, including vaso-occlusive crisis, result in the production of fibrin D-dimer, and its detection may be used as a marker for the presence of the complication.
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THE ROLE OF blood coagulation in the pathogenesis of sickle cell disease is poorly understood. It is, however, known that patients in vaso-occlusive crisis have evidence of intravascular coagulation since fibrinopeptide A levels are elevated in sickle cell crisis.[1] In addition, the activation of coagulation factors may have a profound effect on blood viscosity in sickle cell patients.[2] Sickled erythrocytes adhere to vascular endothelial cells[3,4] and this adherence is enhanced by autologous plasma proteins.[5,6] Therefore, the occlusion of the microvasculature by sickled erythrocytes may be enhanced by interactions with coagulation factors. Sickled erythrocytes[7] or purified spicules from such erythrocytes[8] accelerate blood clotting in vitro. Whether the activation of the coagulation pathway is contributory to vaso-occlusive crisis is unknown. In addition to episodes of painful crisis, many adult patients with sickle cell disease suffer from more chronic complications of the disease, including leg ulcers, aseptic necrosis of bone, and chronic cholecystitis.

To determine if laboratory measurements of fibrinolytic activity are related to the clinical status of the patient and might therefore provide an objective measurement of disease activity, we used a newly available, simple, and rapid latex bead agglutination assay (Dimertest) which is specific for the fragment D-dimer domain in Factor XIII$_a$-crosslinked fibrin. The monoclonal antibody used in the Dimertest, DD-3B6, measures both fragment D-dimer and fragment D-dimer/fragment E complex in plasma.[9] Fragment D-dimer/fragment E complex is a terminal breakdown product of crosslinked fibrin; therefore its presence in plasma is evidence of fibrinolysis. For the purpose of discussion, fibrin D-dimer will refer to the fragment D-dimer domain in all molecular species of crosslinked fibrin and fibrin degradation products. Fibrin D-dimer levels are elevated in fibrinolytic states including disseminated intravascular coagulation, pulmonary embolism, and deep venous thrombosis.[10] We have used this assay to show that there is a strong relationship between fibrin D-dimer levels and disease activity in patients with sickle cell disease.

MATERIALS AND METHODS

Patient samples. EDTA plasma was obtained from blood samples drawn for routine blood count or hemoglobin electrophoresis. Ninety-six patients (68 adults and 28 children) are reported in the present study. Of these, 79 are homozygous for hemoglobin S (Hb SS), ten have Hb SC disease, five have Hb S β^+ thalassemia and one each has Hb SD disease and Hb S β^0 thalassemia. All of these patients have some history of one or more of the complications of sickle cell disease, including vaso-occlusive crisis, leg ulcers, infections (osteomyelitis, pneumonia, viral illness), chronic cholecystitis, aseptic necrosis and bone marrow infarction.

Measurement of fibrin D-dimer levels. To measure the levels of fibrin D dimer in EDTA plasma samples we used the Dimertest latex agglutination assay (American Diagnostica, Greenwich, Conn) according to the manufacturer's recommendations. The minimum amount of fibrin D-dimer detected by the assay is 1 μg/ml.[11] All assays were performed by the same person, who was unaware of the clinical status of the patient. Statistical analysis of the relationship between disease activity and Dimertest results was performed using the G-test of independence.[12]

Assessment of patient clinical status. The patient's clinical status at the time the fibrin D-dimer assays were performed was assessed by history and physical examination. The evaluation was made without knowledge of the assay results. Patients were asymptomatic, were hospitalized in vaso-occlusive crisis, or were having

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other complications of sickle cell disease. Vaso-occlusive crisis was defined as multiple sites of skeletal or other pain lasting at least 12 hours. The other complications of sickle cell disease in these patients included leg ulcers, aseptic necrosis of bone, joint pain, stroke, cholecystitis and infection (pneumonia, osteomyelitis, viral infection).

Biochemical analysis of fragment D-dimer. In order to determine which molecular species of fibrin were recognized by the Dimertest, we analyzed fibrin-related antigens present in EDTA serum. EDTA plasma samples which contained measurable fibrin D-dimer were clotted with thrombin (gift of Dr John W. Fenton II, New York State Department of Health, Albany, NY) and the clot was removed. Fibrinogen-related antigens were immunoprecipitated from serum using rabbit antihuman fibrinogen and Cowan I strain *Staphylococcus aureus* cells (both from Calbiochem-Behring, San Diego) under conditions previously determined to remove all fibrinogen-related antigens. As a control, immunoprecipitations were performed using nonimmune rabbit serum. The precipitates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions and transferred onto nitrocellulose paper by the method of Towbin et al.[13] The blot was probed first with monoclonal antibody DD-3B6 obtained from the enzyme-linked immunosorbent assay (ELISA) form of the Dimertest, and second with ^{125}I-labeled F(ab')$_2$ sheep antimouse immunoglobulin (Amersham, Arlington Heights, Ill). Samples of purified fragment D[14] and fragment D-dimer[15] were electrophoresed in the same gels to serve as internal standards. The blot was then dried and autoradiographed.

RESULTS

Of the 187 samples of EDTA plasma from 96 sickle cell patients tested, 57% had elevated levels of fibrin D-dimer. Normal plasma samples from healthy donors were always negative. When the fibrin D-dimer levels were related to the clinical status of the patients (Fig 1), there was a significant association between disease activity and the presence of fibrin D-dimer ($G = 132.068$, $P < 0.001$). Of 75 samples from 38 asymptomatic patients, only 10% contained measurable levels of fibrin D-dimer. Samples from 14 patients in vaso-occlusive crisis were Dimertest-positive 97% of the time. Samples from six of the patients were taken when the patients were asymptomatic; all of these were Dimertest negative. Among all samples from patients in vaso-occlusive crisis, only one was negative and this patient's diagnosis was not supported by physical findings. In the course of the same hospital admission, this patient experienced chest pain accompanied by a fall in pO_2. A plasma sample drawn 24 hours later contained measurable fibrin D-dimer.

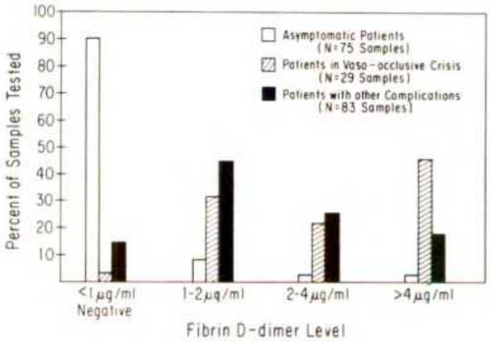

Fig 1. The fibrin D-dimer levels in patients with sickle cell disease grouped according to clinical status. Fibrin D-dimer levels were measured using a latex bead agglutination assay described in Materials and Methods. There is a statistically significant association between elevated D-dimer levels and complications of sickle cell disease ($p < 0.001$).

Eighty-six percent of samples from symptomatic patients not hospitalized for management of vaso-occlusive crisis had elevated levels of fibrin D-dimer. Serial samples were obtained from ten patients in this group who experienced changes in their clinical status other than hospitalization for vaso-occlusive crisis. In all cases, those who developed complications developed measurable levels of fibrin D-dimer while those whose complications resolved became negative in this assay.

There were 14 of the 96 patients (15%) in whom fibrin D-dimer levels did not correlate with clinical symptoms. In eight asymptomatic patients (three adults and five children), fibrin D-dimer levels were elevated while in six symptomatic patients fibrin D-dimer levels were normal. In the latter group, two patients had leg ulcers and four had subjective complaints of pain.

The amount of fibrin D-dimer measured by the agglutination assay was the same in EDTA plasma or serum prepared

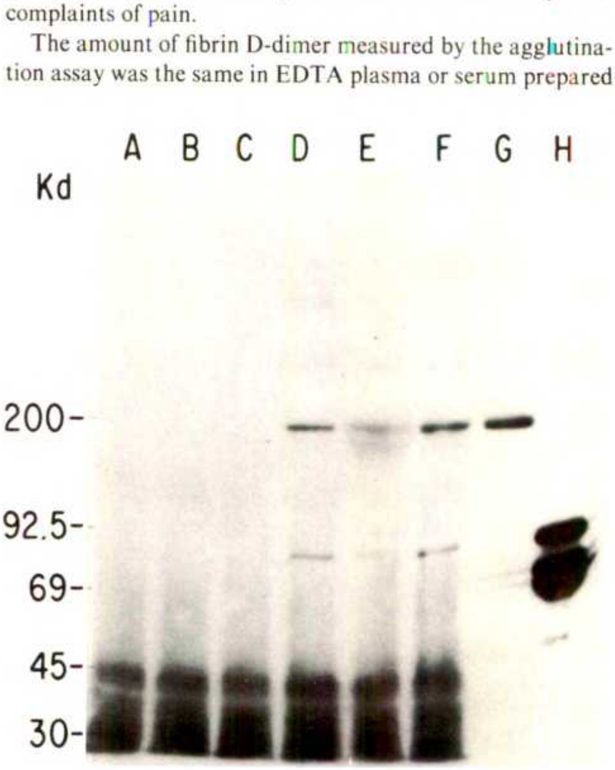

Fig 2. Immunoprecipitation/protein blot analysis of fibrin-related antigens recognized by monoclonal antibody DD-3B6 in plasma samples from sickle cell patients with elevated fibrin D-dimer levels. Control precipitations using nonimmune rabbit serum are found in Lane A (normal donor plasma) and Lane B (Dimertest-positive plasma from a sickle cell patient). As described in the text, fibrin-related antigens were precipitated from serum prepared using thrombin-clotted EDTA plasma samples from a normal donor (Lane C), or Dimertest-positive samples from a sickle cell patient in vaso-occlusive crisis (Lane D) or patients with other complications of sickle cell disease (Lanes E and F). Purified fragment D-dimer and fragment D were electrophoresed in Lanes G and H, respectively. The protein blot was probed with DD-3B6, then radiolabeled F(ab')$_2$ sheep antimouse immunoglobulin.

from thrombin-treated EDTA plasma. Immunoprecipitation/protein blot analysis of the fibrinogen-related antigens in these sera demonstrated that the monoclonal antibody DD-3B6 recognizes primarily fragment D-dimer in addition to some fragment D1 and higher molecular weight fibrin fragments in the Dimertest-positive samples from patients with either vaso-occlusive crisis or other complications of sickle cell disease (Fig 2).

DISCUSSION

There are no practical and readily available laboratory measurements currently in use which correlate with disease state in sickle cell disease. However, there are reports of abnormal coagulation activity in patients with sickle cell disease. Richardson et al[2] found elevated levels of factor VIII:C, factor VIIIR:Ag, and antithrombin III in the plasma of eight patients in steady state which rose higher when the patients were in vaso-occlusive crisis. The onset of vaso-occlusive crisis is also accompanied by an increase in plasma high-molecular-weight fibrinogen complexes (HMWFC).[16] Fibrinopeptide A (FPA) levels have been used to measure coagulation activity in patients in vaso-occlusive crisis. Leichtman and Brewer[1] demonstrated that the FPA level in pain-free patients is generally within normal range but increases with the onset of painful crisis. However, the measurement of both HMWFC and FPA is technically cumbersome and the latter assay is highly susceptible to false positive results due to traumatic venipuncture. Only one of these studies[16] measured coagulation factors in patients with the more chronic complications of sickle cell disease. The single patient with leg ulcers reported showed normal levels of HMWFC.

The measurement of fibrin D-dimer with the latex bead agglutination assay showed significant correlation with disease activity in sickle cell disease. Asymptomatic patients were fibrin D-dimer negative, while patients in vaso-occlusive crisis or patients with other complications of sickle cell disease were positive. Fibrin D-dimer levels may be elevated in conditions other than sickle cell disease. We have found that 28% of 150 random samples from hospitalized patients had measurable levels of fibrin D-dimer.[11] Most of these patients either had diseases associated with an increase in fibrinolytic activity or had recently undergone surgery.

Protein blot analysis of the species of fibrin recognized by the monoclonal antibody in plasma from sickle cell patients demonstrated that while the fragment D-dimer was the predominant species, presumably representing circulating fragment D-dimer/fragment E complexes, some fragment D1 and higher molecular weight forms of fibrin were detected in the blot. The latter may represent fragment DY or fragment Y dimers. Although DD-3B6 recognized fragment D in protein blot analysis, this fragment in fluid phase does not cause agglutination in the Dimertest assay.

The data reported here give no indication of the role abnormal coagulation activation plays in sickle cell disease. It is unclear whether abnormal fibrinolytic activity precipitates the onset of vaso-occlusive events, or is a result of vascular occlusion or vascular injury. However, the close correlation of fibrin D-dimer levels and the patient's clinical condition may provide useful information in the management of patients with sickle cell disease and in the evaluation of therapeutic regimens.

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CORRESPONDENCE

t(1;3)(p36;q21) IN ACUTE NONLYMPHOCYTIC LEUKEMIA: A NEW CLINICOPATHOLOGIC ASSOCIATION

To the Editor:

We agree that t(1;3)(p36;q21) recently reported by Bloomfield et al[1] may prove to be a new cytogenetic-clinicopathologic association in acute nonlymphocytic leukemia (ANLL). This observation should, however, be handled with caution for the following reasons. Weakness and moderately severe anemia do not make up a particularly strong clinical association in a setting such as ANLL, in comparison to associations such as t(8;21) with long-term survival,[2] t(15;17) with intravascular coagulation,[3] and del(16) or inv(16) with high incidence of central nervous system relapse.[4] Furthermore, hematologic findings, such as marrow dyserythropoiesis, should not be considered as a specific hematologic association in view of the fact that ANLL in the adult frequently involves a stem cell that is multipotent for erythrocytes, granulocytes, and platelets[5] and can thus result in variable degrees of dyshematopoiesis.[6]

The most striking finding reported with t(1;3) is dysmegakaryocytopoiesis characterized by normal to increased hypolobulated megakaryocytes. As stated, two other rearrangements of band 3q21 have previously been associated with such findings.[7,8] Furthermore, three other subsets of patients with ANLL have been identified with a similar dysmegakaryocytopoiesis: patients with FAB M4-ANLL,[6] with FAB M7-ANLL,[9] and, finally, with interstitial deletion of the long arms of chromosome 5.[10] Interestingly, two of the three cases reported by Bloomfield et al[1] had a 5q- interstitial deletion. It appears difficult to convincingly attribute the dysmegakaryocytopoietic status observed in those two patients to the 3q21 rearrangement alone. In conclusion, we believe that the criteria put forward by Bloomfield et al[1] in favor of this new cytogenetic-clinicopathologic association in ANLL are not specific enough. As suggested by the authors in their discussion, t(1;3)(p36;q21) should, at the present time, be considered as a variant form of rearrangement affecting band 3q21. Megakaryocytic proliferation and maturation appear to be under the control of complex genetic regulation. Molecular cloning of the 3q21 breakpoint may reveal valuable information on the molecular basis of megakaryocytic regulation.

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To the Editor:

Gyger questions whether the t(1;3)(p36;q21) represents a specific clinicopathologic subset of acute nonlymphocytic leukemia (ANLL). Additional carefully studied cases will have to be reported before we can conclusively respond. However, our prior experience[1-4] suggests that once an entity is recognized and reported, others begin to find the entity, and thus delineation of its precise features becomes possible. Indeed, this already seems to be happening for the t(1;3). Since initial recognition of the entity by Moir et al,[5] there have already been at least 12 additional patients reported, and each month seems to bring new reports.[6-14] Unfortunately, the data reported on many of the patients are minimal. It is our hope that as a result of our detailed report,[8] others will comment on the recurring features we have found so we can see how characteristic they are.

Table 1 highlights a number of features found in the 14 patients for whom data are available. Nine had the t(1;3) as the sole cytogenetic abnormality; in four patients, additional abnormalities occurred, including a deletion 5q in three. The clinical picture seems to be similar regardless of whether there are additional chromosomal abnormalities. Most of the cases were difficult to classify according to French-American-British (FAB),[15,16] as is often the case in leukemias involving all cell lines (panmyeloses). A myelodysplastic syndrome was first diagnosed in six patients; in three of these, acute transformation was documented. A long preleukemic phase was documented in two additional patients. A history of prior chemotherapy or radiotherapy was found in only two patients; one had been treated for breast cancer and the other for polycythemia vera. There is a slight female preponderance, which is unusual in "carcinogenic" exposure-related leukemias. However, among the six males reported, one was a brick-factory worker, one a mason, and a third had spent the prior two years building his home. From an etiologic standpoint,

Table 1. Clinicopathologic Features at Time of Chromosome Study of 14 Patients With t(1;3)(p36;q21)

Age/ Sex	Diagnosis*	Prior Exposure	Documented Preleukemic Phase	Evolution to ANLL	Hemoglobin (g/dL)	Platelets ($\times 10^9$/L)	WBC ($\times 10^9$/L)	Response to ANLL Therapy	Additional Karyotype Abnormalities	Reference
40F	RA	—	NA	–	7.6	262	4		None	5
80M	?RAS	?	NA	?	?	658	?		?	7
37M	?CMML	—	NA	+	4.8	567	5		None	5
49F	CMML	—	NA	–	?	?	?		None	10
70F	RAEB-T	—	NA	+	6.1	135	3	F	None	9
37F	RAEB-T	—	NA	+	7.2	292	6		del(12)(p13), del(15)(q21)	9
73F	ANLL	P32	P.vera	NA	9.2	552	7	F	del(5)(q32)	6
56F	ANLL	—	+	NA	↓	481	6		None	9
36M	ANLL-M1	—	–	NA	8.0	95	96		del(5)(q12q32)	8
47F	ANLL-M1	CT & RT	–	NA	9.4	233	2	F	del(5)(q31q32)	8
61M	ANLL-M4	Brick factory	–	NA	7.0	220	3	F	None	8
54M	ANLL-M4	Mason	–	NA	6.5	↓	5	F	None	11
49M	ANLL-M4	—	+	NA	10.0	300†	53	F	None	12
14F	ANLL-?M4	—	–	NA	7.5	21	69	CR 6 mo	None	5

RAS, RA with ring sideroblasts; NA, not applicable; CT, chemotherapy; RT, radiotherapy; F, fail, no remission; CR, complete remission; ?, data not given; ↓, decreased; +, present; –, absent.

*To the extent possible, diagnosis is FAB.[15,16]

†Five months before chromosome study. Not given at time of chromosome study.

it is also of interest that we know of no cases reported from the United States.

Anemia (median hemoglobin, 7.5 g/dL) was present in all patients for whom data were provided. It was much more severe than reported in most large series of ANLL (University of Minnesota median, 9.5). The other striking hematologic feature at diagnosis was a high platelet count (median, 262 × 10^9/L); the median at the University of Minnesota is 62 × 10^9/L. Finally, response to chemotherapy has been poor. Seven patients have had intensive chemotherapy for ANLL; only one had a brief complete remission. Only two of the five failures had additional chromosomal abnormalities.

These data suggest to us that t(1;3)(p36;q21) does indeed identify a specific subtype of myeloid leukemia. It appears to be characterized by being a panmyelosis often difficult to classify according to FAB. Patients tend to present with low hemoglobins and high platelet counts, and response to standard ANLL therapy is poor. The importance of additional chromosomal abnormalities is unknown, but clearly the deletion 5q does not account for the above syndrome, since it occurred in only three of the patients. Which aspects of the syndrome, if any, are accounted for solely by the break in 3q21 is also unclear; Rowley's group has suggested that both a break at 3q21 and 3q26 are required for platelet abnormalities in situations other than t(1;3).[17]

How characteristic the megakaryocytic abnormalities we reported are is unknown, because others have not described the morphology in detail. Further cases must be studied to increase our understanding of this clinicopathologic entity, but for the present, the t(1;3)(p36;q21) seems to clearly identify an unusual subgroup of ANLL.

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SERUM LACTIC DEHYDROGENASE ISOENZYME PATTERN IN CHILDHOOD LYMPHOBLASTIC LEUKEMIA

To the Editor:

We have read with great interest the recent article by Pui et al[1] describing the prognostic value of serum lactic dehydrogenase (LDH) levels in childhood acute lymphoblastic leukemia (ALL). Their results confirm our findings.[2]

In our study, in addition to elevation of serum LDH levels, isoenzyme patterns of LDH were also related to the prognosis of childhood ALL. The highest concentration of LDH-3 was observed in the high-risk group at the time of diagnosis. The ratio of LDH-3 to LDH-2 was found to be greater than 1.0 in more than 70% of children in the high-risk group but in none of the patients in the standard-risk group.

We believe that in addition to serum LDH values, the determination of its isoenzymes would be helpful in the identification of a high-risk group of children with ALL.

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To the Editor:

We are interested in the confirmatory finding of Hiçsönmez et al that children with acute lymphoblastic leukemia (ALL) and high serum lactic dehydrogenase (LDH) levels have poor prognosis features at diagnosis. We have recently studied the isoenzyme pattern of serum LDH in 29 children with ALL. Using the identical criteria to define risk groups,[1] in contrast to the findings of Hiçsönmez et al, we found no difference in the distribution of serum LDH isoenzymes between high- and standard-risk patients (see Table 1). None of the patients in the standard-risk group and only one in the high-risk group had a serum LDH-3-LDH-2 ratio over 1.0 at presentation ($P = 1.0$ by two-tailed Fisher exact test). The reason for this discrepancy is unclear but may be related in part to our use of fresh serum samples, as opposed to frozen samples in their study.[2] Although serum LDH level was found to have independent prognostic value, the pathophysiologic basis for an increase of serum LDH is poorly understood.[3]

Table 1. Distribution of Serum LDH Isoenzymes According to Risk Group

Feature	No. of Patients	Percentage (Mean ± SD) of Total Activity				
		LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
High risk	21	12.4 ± 4	42.8 ± 6	28.9 ± 7	11.1 ± 3	5.2 ± 5
Standard risk	8	16.0 ± 3	47.6 ± 4	22.2 ± 4	9.6 ± 3	4.6 ± 1

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ERRATUM

In the article entitled "Use of Oligonucleotide Hybridization in the Characterization of a β-Thalassemia Gene (β^{37}TGG → TGA) in a Saudi Arabian Family" by Boehm et al, which appeared in the April 1986 issue (Volume 67, Number 4, page 1185), Figure 2B was omitted. Figure 2 is reprinted in its entirety below.

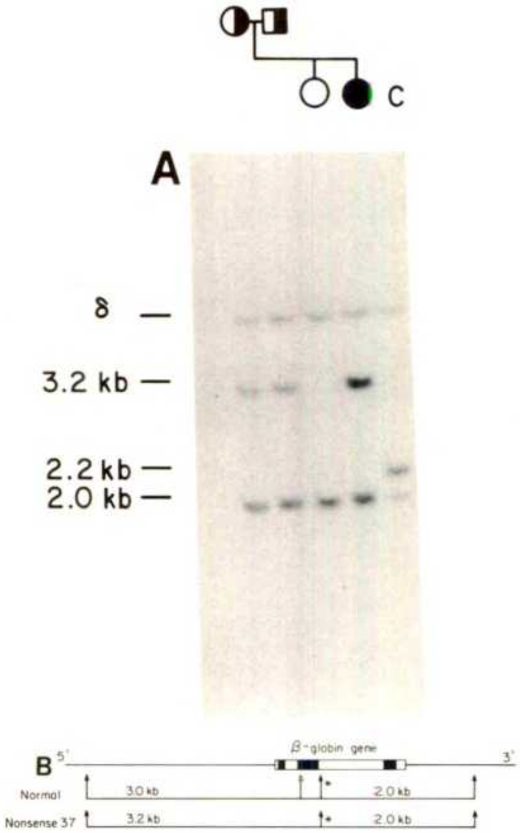

Fig 2. (A) Autoradiogram of *Ava*II-digested genomic DNA from family members indicated by the pedigree. β-Globin cDNA (JW 102) was used as the probe. The two alleles of an *Ava*II polymorphism in IVS 2 of the gene appear as 2.2-kb and 2.0-kb fragments on the autoradiogram. All family members are homozygous for the presence of the polymorphic site (+/+), and the control (c) is heterozygous +/−. A new band at 3.2 kb is seen in this family and is segregating with the β-thalassemia allele. The appearance of this fragment indicates loss of a nonpolymorphic *Ava*II site in exon 2 of the β-globin gene. Probe cross hybridizes with δ-globin sequences. (B) *Ava*-II restriction map of a normal β-globin gene and the mutant β-globin gene described in the text. Solid arrows (↑) indicate the locations of the nonpolymorphic *Ava*II restriction sites within and flanking the β-globin gene. Solid arrows with asterik (↑•) indicate the location of a polymorphic *Ava*II site, which is present in both the mutant and normal β-globin genes. Open arrow (⇡) indicates the location of the nonpolymorphic *Ava*II site, which is destroyed in the mutant gene. The 3.0-kb fragment is not seen in the autoradiogram in Fig 2a because the 5' end of the JW 102 β-globin cDNA probe is complementary to only about 50 bp of this fragment.

ANNOUNCEMENTS

RECENT ADVANCES IN LEUKEMIA AND LYMPHOMA

Organizers: Robert Peter Gale and David W. Golde

January 25–31, 1987;* Keystone, Colorado

Dramatic advances have been made during the last five years in the pathogenesis and basic biology of the leukemias and lymphomas. Research involving normal and neoplastic hematopoiesis and the molecular basis of hematopoietic cell transformation is at the very forefront of our understanding of human oncogenesis and the clinical treatment of human cancer. Key issues to be addressed at this meeting include abnormal gene expression and regulation of oncogenes and related genes, interrelationships between genetic abnormalities and hematopoietic growth factors, and the role of viruses in experimental and clinical leukemias and lymphomas. We also will consider current concepts of hematopoietic and lymphoid cell growth and development and the role of immunology in the diagnosis and therapy of these disorders. Therapeutic advances such as new drugs, treatment schedules, and bone marrow transplantation with and without *in vitro* treatment will be evaluated. New strategies such as gene transfer in hematopoietic cells will be discussed in the context of the biology of the leukemias, the introduction of drug resistance genes, and the correction of leukemia-specific genetic abnormalities. The integration of studies of basic biology, clinical features, and therapeutic advances will serve to summarize critically the current state of this rapidly evolving field and to develop future research strategies. Plenary session topics include: Chromosomes, DNA, and Leukemia; Oncogenes in Leukemia/Lymphoma; Viruses and Leukemia/Lymphoma; Growth Factors and Receptors; Hematopoietic Stem Cells and Differentiation; Immunology of Leukemia and Lymphoma; Gene Transfer into Hematopoietic Stem Cells; Therapy.

Related 1987 UCLA Symposia include: GROWTH REGULATION OF CANCER, January 17–23,* Park City, Utah; TUMOR PROGRESSION AND METASTASIS and ALTERED GLYCOSYLATION IN TUMOR CELLS (concurrent), April 6–12,† Keystone, Colorado; and THE T CELL RECEPTOR, April 12–17,† Keystone, Colorado.

Abstract Submission Deadlines: *, October 3, 1986; †, December 12, 1986. Applications will be accepted at any time for meetings which are not over-subscribed. *For further information,* please call or write: UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, CA 90024. Telephone: (213) 206-6292. Telex: UCLA Symposia 9103427597.

CONTEMPORARY TOPICS IN EXPERIMENTAL HEMATOLOGY

A Symposium in conjunction with the

15TH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY OF EXPERIMENTAL HEMATOLOGY

August 10–14, 1986

Hyatt Regency Hotel, Buffalo, New York

PRESENTERS

Peter J. Quesenberry, MD, Charlottesville, Va.
Rainer Storb, MD, Seattle, Wash.
Bob Löwenberg, MD, Rotterdam, The Netherlands
Harvey D. Preisler, MD, Buffalo, NY
Shiman Slavin, MD, Jerusalem
Ronald Hoffman, MD, Indianapolis, Ind.
Jun Minowada, MD, Okayama, Japan
Malcolm S. Mitchell, MD, Los Angeles, Calif.
David W. Golde, MD, Los Angeles, Calif.
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Selected Topics of the ISEH meeting: Stroma; Bone Marrow Transplantation; Growth Factors; Differentiation; Stem Cells; Myeloproliferative Disorders; Leukemia/Lymphoma.

Sponsored by the National Cancer Institute, the National Heart, Lung, and Blood Institute, and the National Institutes of Health. Fees: $160.00 for ISEH members; $185.00 for nonmembers. Registration for the ISEH meeting will be held August 9–10, 1986, at the Hyatt Regency Hotel. *For further information and preregistration,* contact M.P. McGarry, MD, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263. Preregistration ends July 15.

Blood, Vol 68, No 1 (July), 1986: pp 324–325

WILMOT CANCER RESEARCH FELLOWSHIPS

The University of Rochester School of Medicine offers research training to physicians who have completed at least one year of residency or research experience. Fellowships will begin July 1, 1987. Opportunities exist in any discipline relevant to the cause, diagnosis, treatment, or prevention of cancer, including endocrinology, experimental therapeutics, genetics, hematology, immunology, microbiology, molecular and cellular biology, pathology, and radiation oncology.

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For further information, contact: Wilmot Cancer Research Fellowship Program, Medical Center Box 601, University of Rochester School of Medicine & Dentistry, 601 Elmwood Avenue, Rochester, New York USA 14642.

INTENSIVE REVIEW OF HEMATOLOGY-ONCOLOGY

Boston, Massachusetts

October 20–24, 1986

Sponsored by the Hematology Division, Brigham and Women's Hospital, and Harvard Medical School. *For further information, contact:* Harvard Medical School, Department of Continuing Education, Boston, MA 02115. Telephone: (617)732-1525.

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The New York Academy of Sciences

BLOOD IN CONTACT WITH NATURAL AND ARTIFICIAL SURFACES

November 12-14, 1986

The Sheraton Centre, New York City

The purpose of this conference is to unite scientists from diverse disciplines, such as physical chemistry, the engineering sciences, biology and medicine to participate in a dialogue on the present status of surfaces and their relationship to thrombosis. This meeting will range from didactic presentations of basic concepts common to specific disciplines to state-of-the-art exchanges of ideas and methodologies among scientists studying the interaction of blood with prosthetic and naturally-derived surfaces.

There will be contributed poster sessions. The deadline for the submission of abstracts is July 31, 1986. A 200-word summary of the work to be presented should be sent to: Dr. Vincent Turitto, Mount Sinai Medical Center, Division of Cardiology/23rd Floor, 1 Gustave Levy Place, New York, NY 10029.

Chairs: Vincent Turitto, ScD., Mount Sinai Medical Center, NY; Edward Leonard, Ph.D., Columbia University, NY and; Leo Vroman, Ph.D., Brooklyn VA Hospital, NY.

Inquiries should be addressed to: Conference Department, The New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021.

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BLOOD BANK DIRECTOR

The American Red Cross Blood Services, Rochester Region, is seeking a blood bank director with an M.D. The applicant must have experience in blood banking and administration, have an interest in research and teaching, and be qualified for an academic appointment of the faculty of the University of Rochester School of Medicine and Dentistry.

The American Red Cross Blood Services, Rochester region provides all the blood products for 43 hospitals in a 15-county area. It collects 100,000 units of blood per year and produces 250,000 blood products. The director of Blood Services is responsible for a program staff of 170. The director interacts with leadership volunteers on a regular basis

Interested candidates should submit their curriculum vitae to: ***CHAIRPERSON, Search Committee, ℅ Personnel Department, American Red Cross, 50 Prince Street, Rochester, New York 14607.***

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[1] Rubin, L. A., C. C. Kurman, M. E. Fritz, W. E. Biddison, B. Boutin, R. Yarchoan, and D. L. Nelson. 1985. Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. **The Journal of Immunology** 135:3172.

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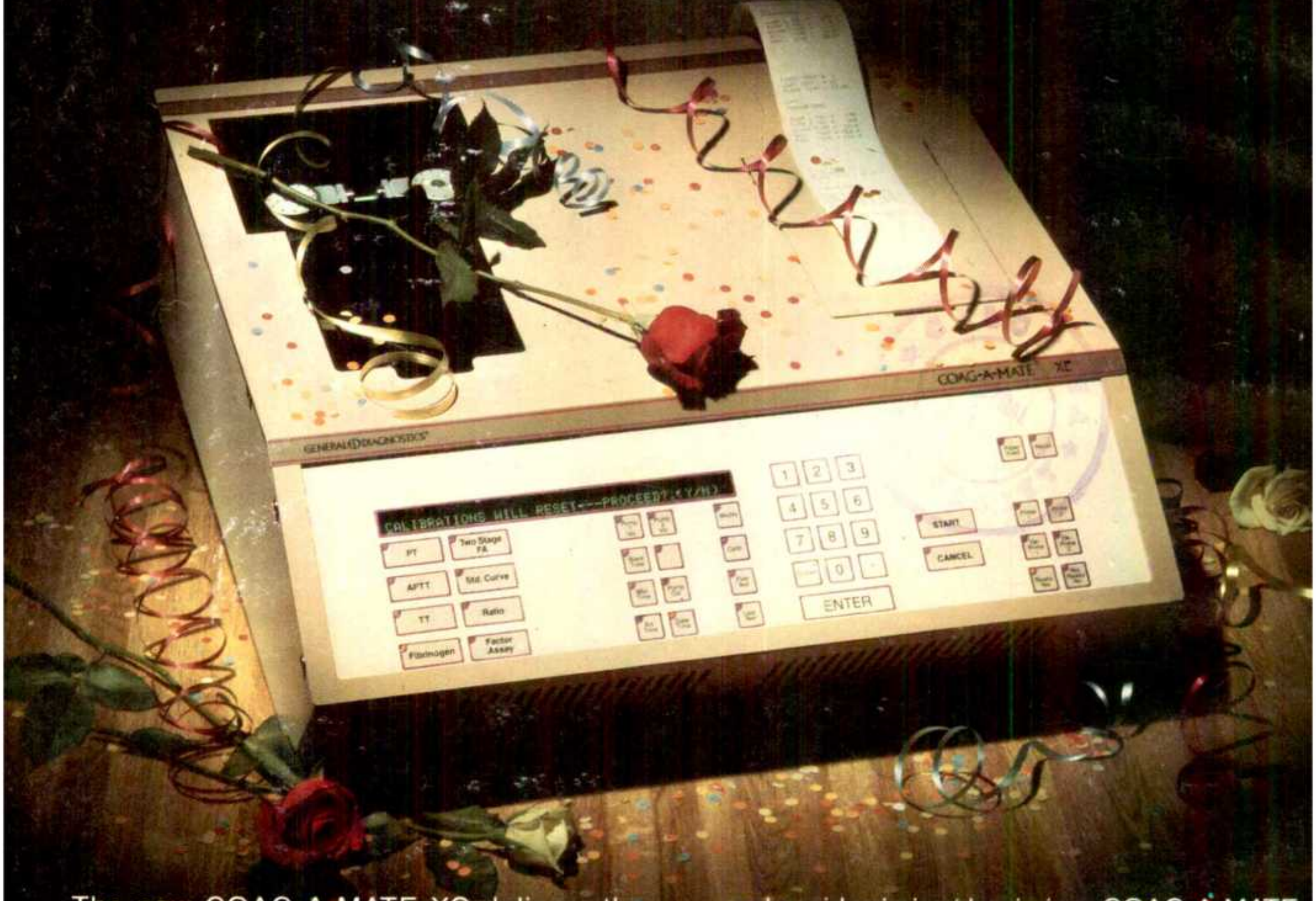

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CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

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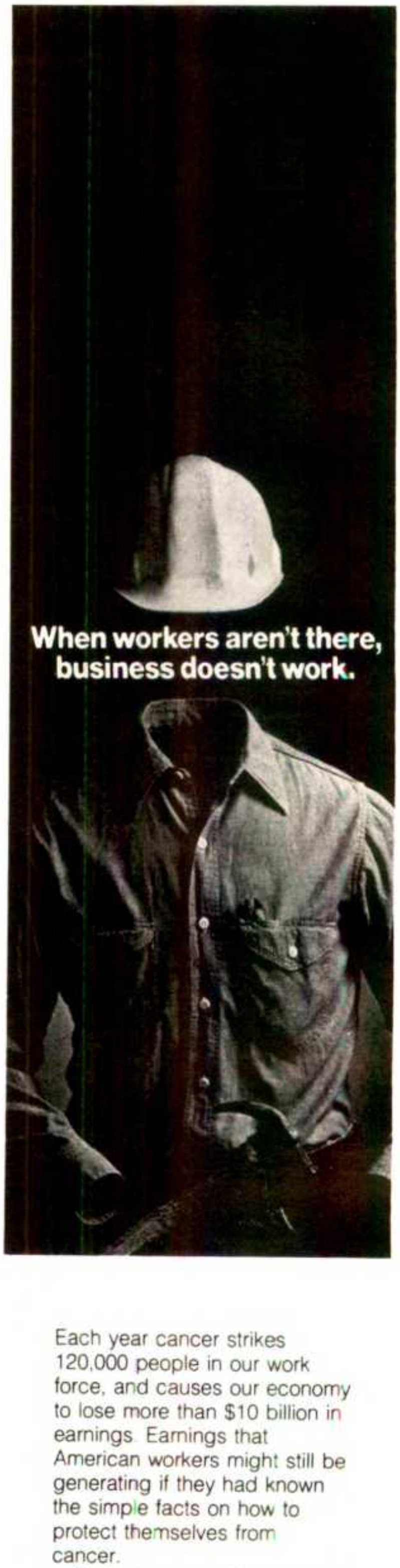

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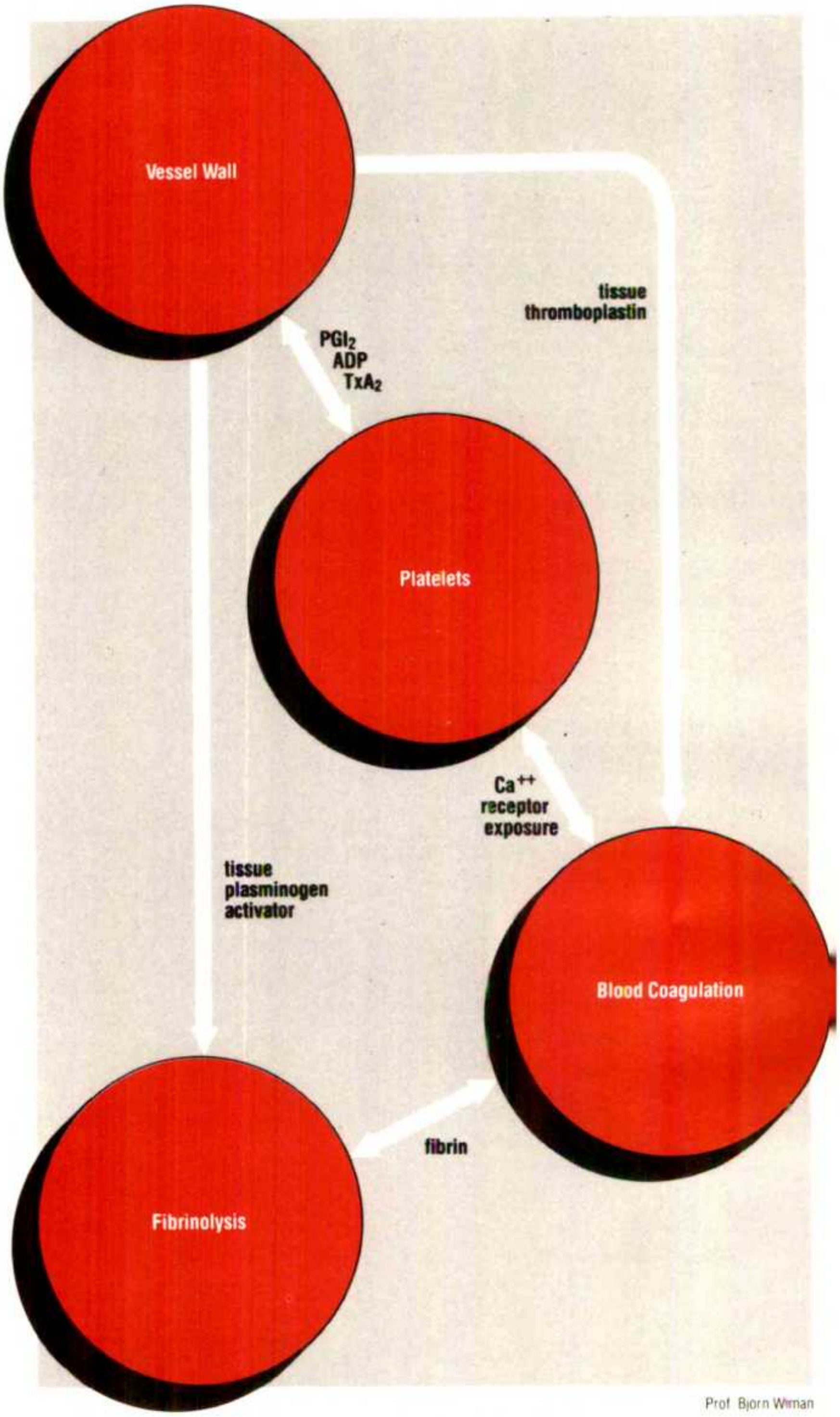

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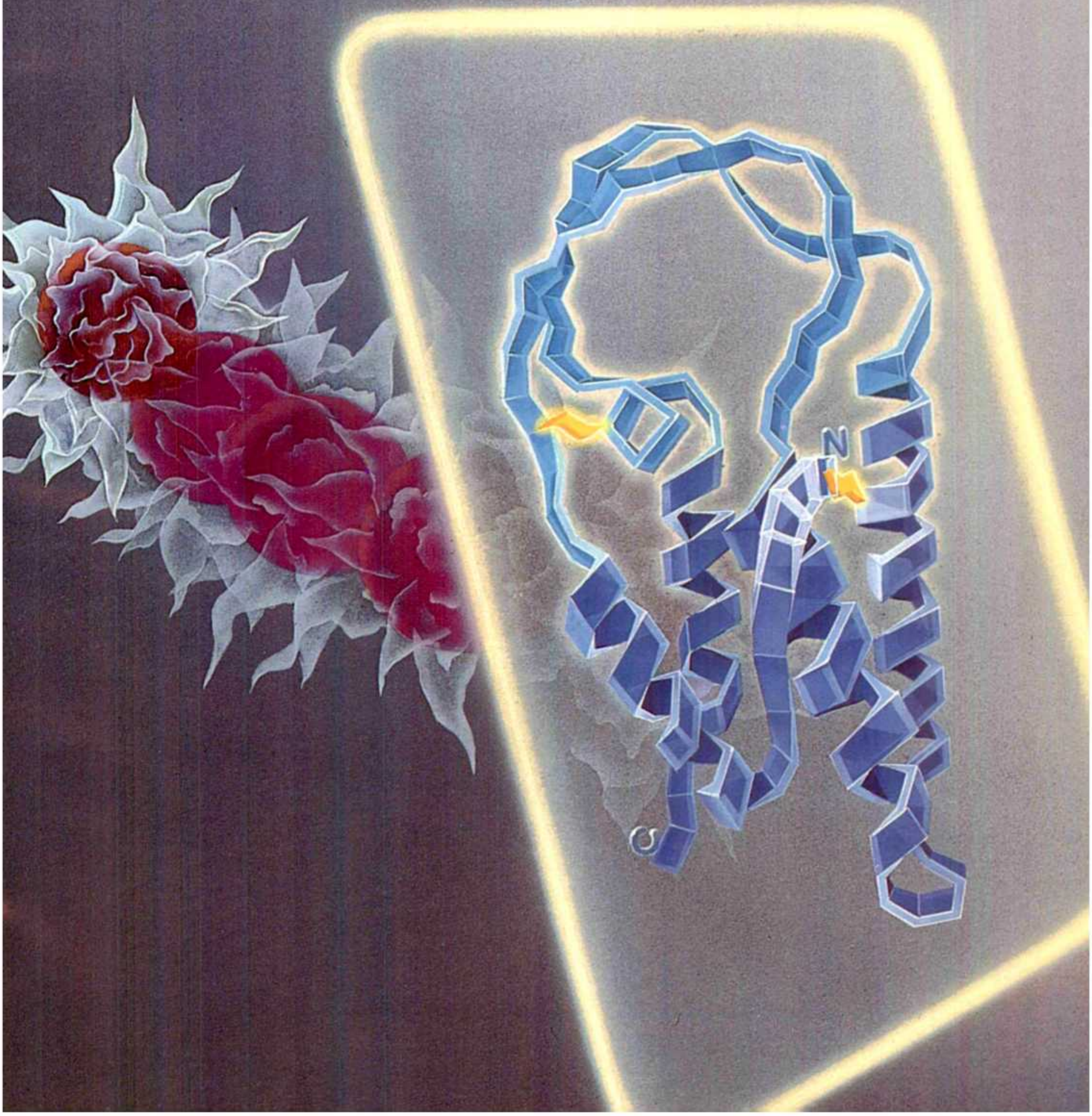

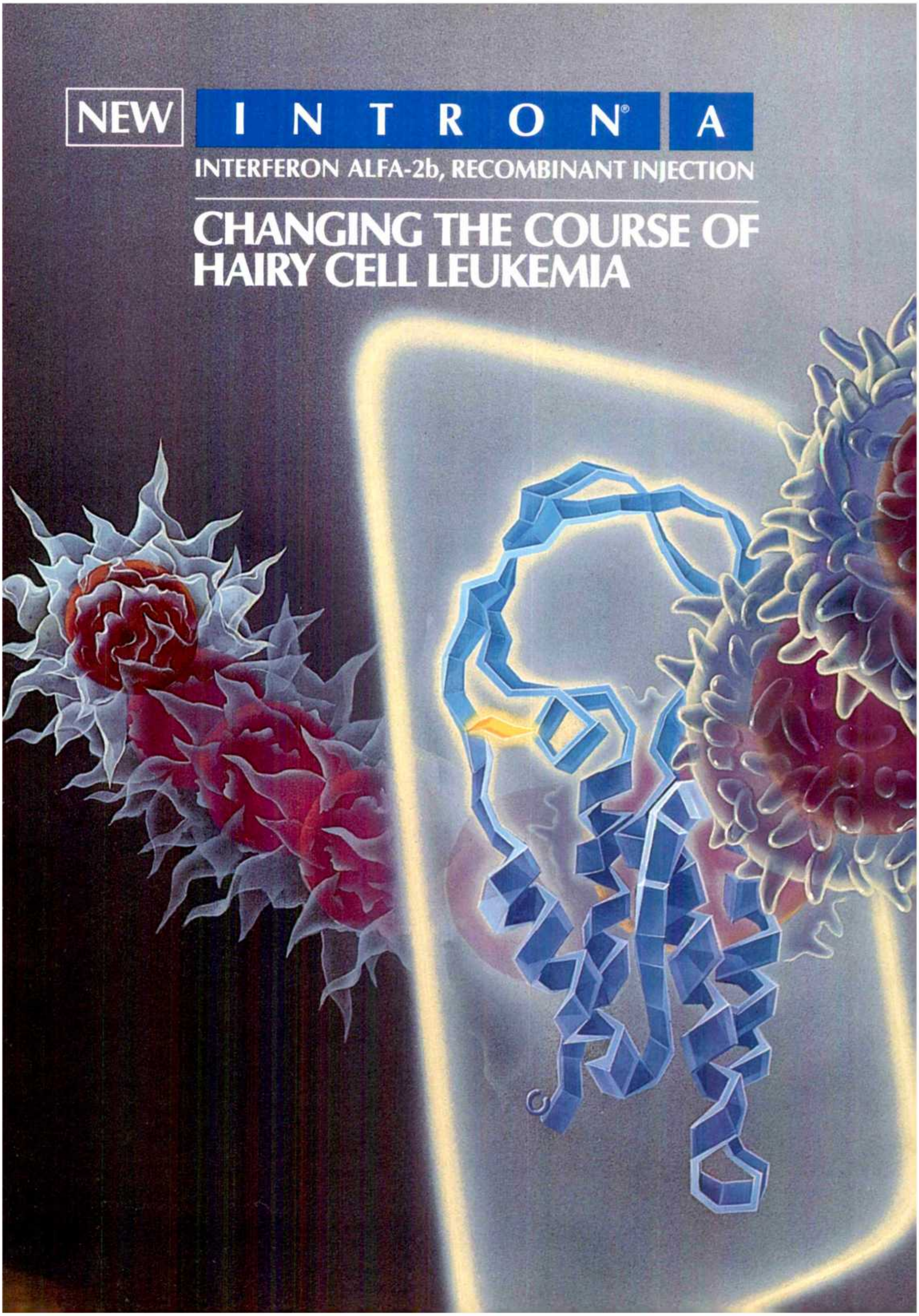

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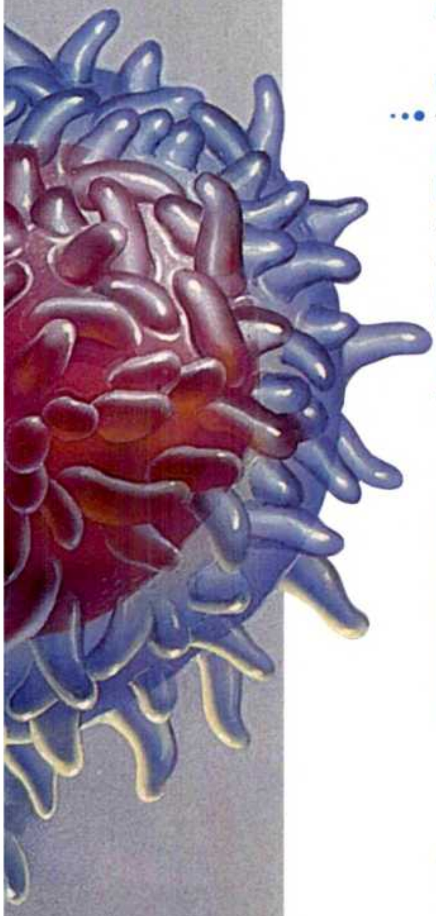

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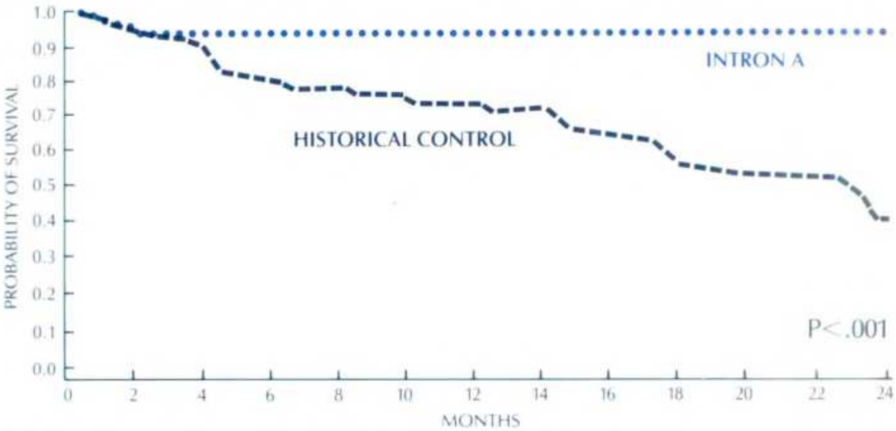

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*Seventy-one historical control patients were treated with current standard therapies, 85% with chemotherapy (mainly chlorambucil) and 15% with supportive care.

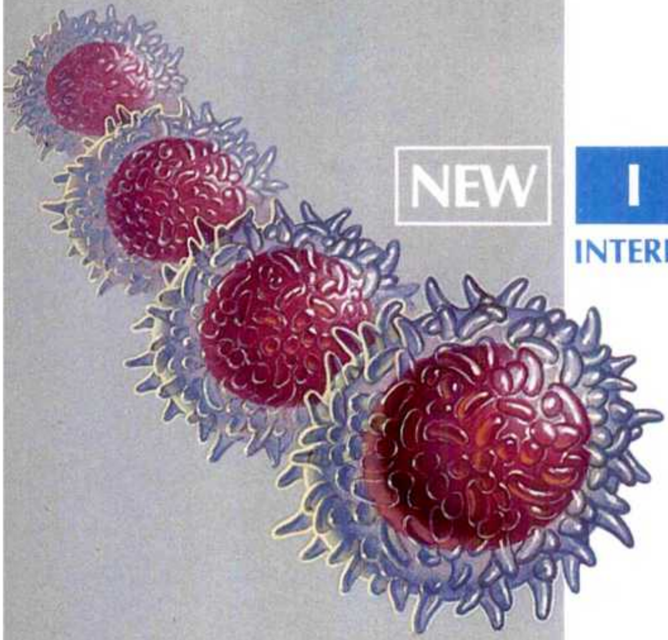
DRAMATIC CLINICAL IMPROVEMENTS

More than 80% of the study population achieved normal levels of platelets by three months, of hemoglobin by six months and of granulocytes by twelve months. Marked decreases in the incidence of life-threatening infections and transfusion dependency preceded normalization of hematologic values.[1]

THERAPY WELL TOLERATED

Treatment-related side effects were generally mild to moderate and rapidly reversible upon discontinuation of therapy. The predominant complaint was a flu-like syndrome, which usually diminished with continued therapy. Mild-to-moderate skin rashes occurred intermittently in 40% of the study population. A low incidence of mild-to-moderate GI disorders was also reported.[1]

For a brief summary of prescribing information, please see the following page.



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BRIEF SUMMARY

INDICATIONS AND USAGE INTRON A for Injection is indicated for the treatment of patients 18 years of age or older with hairy cell leukemia. Studies have shown that INTRON A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and non-splenectomized patients.

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically during treatment to determine whether response to treatment has occurred. If a patient does not respond within 6 months, treatment should be discontinued. If a response to treatment does occur, treatment usually should be continued until no further improvement is observed and these laboratory parameters have been stable for about 3 months (see **DOSAGE AND ADMINISTRATION**). It is not known whether continued treatment after that time point is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS INTRON A is contraindicated in patients with a history of hypersensitivity to interferon alfa or any component of the injection.

WARNINGS Moderate to severe adverse experiences may require modification of the patient's dosage regimen, or in some cases, termination of therapy with INTRON A.

Because of the fever and other "flu-like" symptoms associated with INTRON A administration, it should be used cautiously in patients with debilitating medical conditions, such as those with a history of cardiovascular disease (e.g., unstable angina, uncontrolled congestive heart failure), pulmonary disease (e.g., chronic obstructive pulmonary disease), or diabetes mellitus prone to ketoacidosis. Caution should also be observed in patients with coagulation disorders (e.g., thrombophlebitis, pulmonary embolism) or severe myelosuppression.

Patients with platelet counts of less than 50,000/mm^3 should not be administered INTRON A intramuscularly, but instead by subcutaneous administration.

Cardiovascular adverse experiences which include significant hypotension, arrhythmia, or tachycardia of 150 beats per minute or greater, were observed in approximately 3% of the patients studied who had various malignancies and were treated at doses higher than those for hairy cell leukemia. The incidence of these complications in patients with preexisting heart disease is unknown. Hypotension may occur during administration, or up to two days post therapy, and may require supportive therapy including fluid replacement to maintain intravascular volume. Supraventricular arrhythmias occurred rarely and appeared to be correlated with preexisting conditions, and prior therapy with cardiotoxic agents. These adverse experiences were controlled by modifying the dose or discontinuing treatment, but may require specific additional therapy.

Those patients with a recent history of myocardial infarction and/or previous or current arrhythmic disorder, who require INTRON A therapy, should be closely monitored (see **Laboratory Tests**).

Central nervous system effects manifested by depression, confusion and other alterations of mental status were observed in about 2% of hairy cell leukemia patients treated with INTRON A. The overall incidence in a larger patient population with other malignancies treated with higher doses of INTRON A was 10%. More significant obtundation and coma have been observed in some patients, usually elderly, treated at higher doses for other malignant diseases. These effects are usually rapidly reversible. In a few severe episodes, full resolution of symptoms has taken up to three weeks. Patients should be closely monitored until resolution of these effects. Discontinuation of INTRON A Interferon alfa-2b, recombinant for Injection therapy may be required. Narcotics, hypnotics, or sedatives may be used concurrently with caution.

Acute serious hypersensitivity reactions (e.g., urticaria, angioedema, bronchoconstriction, anaphylaxis, etc.) have not been observed in patients receiving INTRON A; however, if such an acute reaction develops, the drug should be discontinued immediately and appropriate medical therapy instituted. Transient cutaneous rashes have occurred in some patients following injection, but have not necessitated treatment interruption.

Laboratory abnormalities which occurred in hairy cell leukemia patients included elevated SGOT and SGPT, which occurred in 4% and 13% of patients, respectively. The overall incidences of laboratory abnormalities in a larger patient population with other malignancies, and treated at higher doses were somewhat higher. These included elevated liver function tests (SGOT, SGPT in 10% of patients) and reductions in granulocyte (20% of patients) and platelet counts (18% of patients) (see **Laboratory Tests**). These abnormalities are usually mild to moderate and transient. Severe abnormalities of these laboratory parameters are usually rapidly reversible upon cessation or reduction of INTRON A therapy.

PRECAUTIONS Information for Patients Patients being treated with INTRON A should be directed in its appropriate use, informed of benefits and risks associated with treatment and referred to the Patient Information Sheet.

Patients should be cautioned not to change brands of Interferon without medical consultation as a change in dosage may result.

It is advised that patients be well hydrated especially during the initial stages of treatment.

Laboratory Tests In addition to those tests normally required for monitoring patients with hairy cell leukemia, the following laboratory tests are recommended for all patients on INTRON A therapy, prior to beginning treatment and then periodically thereafter:

- Standard hematologic tests — including complete blood counts and differential as well as platelet counts.
- Blood chemistries — electrolytes and liver function tests.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer, should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis, Impairment of Fertility Studies with INTRON A have not been performed to determine carcinogenicity, or the effect on fertility. Interferon may impair fertility. Mutagenicity studies with INTRON A revealed no adverse effects.

Studies in mice, rats, and monkeys injected with INTRON A for up to one month have revealed no evidence of toxicity. However, due to the known species-specificity of interferon, the effects in animals are unlikely to be predictive of those in man.

Pregnancy Category C Animal reproduction studies have not been conducted with INTRON A. It is also not known whether INTRON A can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. INTRON A should be given to a pregnant woman only if clearly needed. Another interferon alfa preparation has been shown to have abortifacient effects in *Macaca mulatta* (rhesus monkeys) when given at 20 to 500 times the human dose. Therefore, INTRON A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. In studies of interferon use in non-human primates, abnormalities of the menstrual cycle have been observed.

INTRON A Interferon alfa-2b, recombinant for Injection should be used with caution in fertile men and women.

Nursing Mothers It is not known whether this drug is excreted in human milk. However, studies in mice have shown that mouse interferons are excreted into the milk. Because of the potential for serious adverse reactions from INTRON A in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use Safety and effectiveness have not been established in patients below the age of 18 years.

ADVERSE REACTIONS Adverse reactions to INTRON A are dose-related. In studies of patients with malignancies other than hairy cell leukemia, hematologic and hepatic toxicities were more common in patients receiving individual injections greater than 10 million IU, than in patients receiving less than this amount. Hematologic, hepatic, cardiovascular and neurologic toxicities were more common in patients receiving more than 30 million IU per injection than in patients receiving less than that amount.

The following adverse reactions were reported to be possibly or probably related to INTRON A therapy during clinical trials. The most frequently reported adverse reactions were flu-like symptoms, primarily fever, fatigue, and chills which occurred in almost all patients treated. Other organ systems in which reactions occurred in more than 5% of the patients are:

General — Taste alteration, anorexia and weight decrease occurred uncommonly.

Cardiovascular — Mild hypotension was frequently recorded although it was rarely symptomatic. Hypertension also occurred less commonly. Tachycardias occurred uncommonly usually in association with high fevers.

Gastrointestinal System — Nausea was common, although vomiting occurred rarely. Mild diarrhea was reported less commonly.

Hematologic — Mild thrombocytopenia and transient granulocytopenia occurred commonly.

Musculo-Skeletal System — Myalgia and arthralgia occurred commonly, usually in association with other flu-like symptoms.

Nervous System — Headache, somnolence, confusion, and dizziness occurred uncommonly. Ataxia and paresthesia were rare.

Psychiatric — Anxiety, depression and nervousness occurred uncommonly.

Skin and Appendages — Mild pruritus occurred uncommonly. Mild alopecia was uncommon. Various transient, mild skin rashes were seen frequently in patients with hairy cell leukemia.

Other Adverse Reactions — The following adverse reactions were reported with an incidence of 5% or less: leg cramps, constipation, insomnia, herpetic eruptions, non-herpetic cold sores, urticaria, hot flashes, supraventricular arrhythmias, epistaxis, stomatitis, paralytic ileus, dehydration, coagulation disorder (elevated PT and PTT), abnormal vision, tremor, emotional lability, chest pain, pharyngitis and syncope.

Adverse reactions reported rarely (less than 1%) were dyspepsia, purpura, dyspnea, sneezing, oculomotor paralysis, nasal congestion, flatulence, increased saliva, hyperglycemia, and ulcerative stomatitis.

Laboratory Value Changes — Those patient laboratory values which were normal to moderately abnormal (WHO grades 0–2) at baseline that worsened to either severe or life-threatening abnormalities (WHO grades 3 or 4) during some phase of treatment included: WBC, platelets, granulocytes, SGOT, creatinine, SGPT, LDH, and alkaline phosphatase.

DOSAGE AND ADMINISTRATION The recommended dosage of INTRON A for Injection for the treatment of hairy cell leukemia is 2 million IU/m^2 administered intramuscularly (see **WARNINGS**) or subcutaneously 3 times a week. Higher doses are not recommended. The normalization of one or more hematologic variables usually begins within 2 months of initiation of therapy. Improvement in all three hematologic variables may require 6 months or more of therapy.

This dosage regimen should be maintained unless the disease progresses rapidly, or severe intolerance is manifested. If severe adverse reactions develop, the dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. If persistent or recurrent intolerance develops following adequate dosage adjustment, or disease progresses, treatment with INTRON A Interferon alfa-2b, recombinant for Injection should be discontinued. The minimum effective dose of INTRON A has not been established.

At the discretion of the physician, the patient may self-administer the dose. The medication may be administered at bedtime.

HOW SUPPLIED INTRON A Interferon alfa-2b, recombinant for Injection is available in 3-, 5-, 10- and 25-million IU vials, each packaged with its own diluent.

Store INTRON A for Injection, both before and after reconstitution, between 2° and 8°C (36° and 46°F).

JBS-13762805

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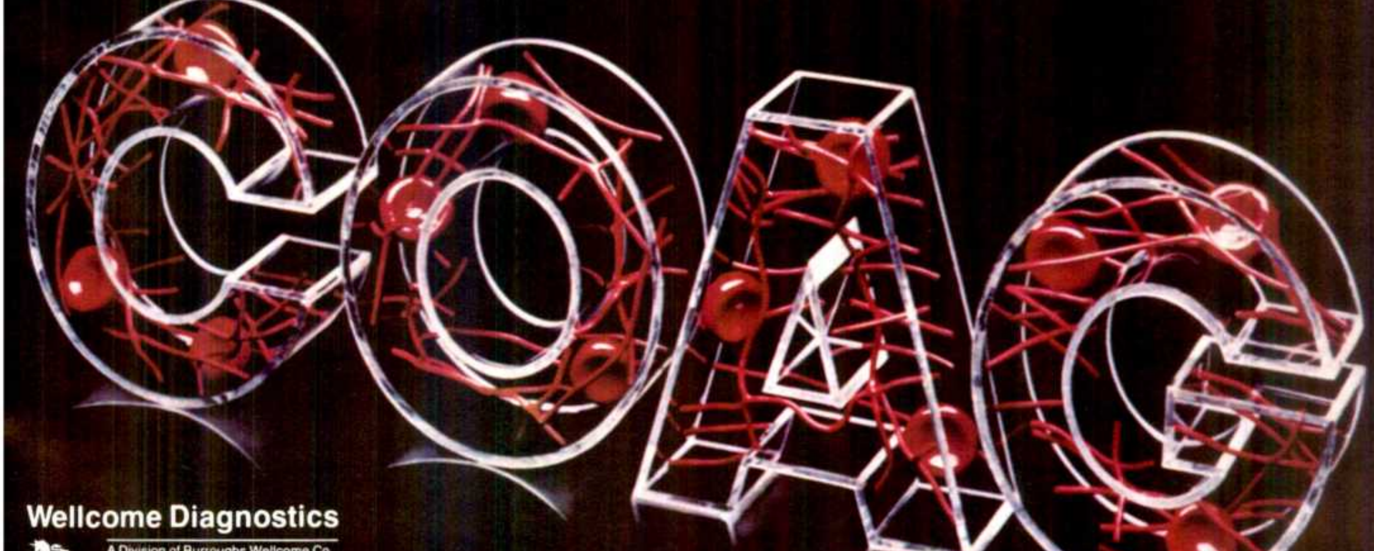

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All checks and money orders must be marked with the first author's name, in order to avoid delay in processing. Receipt of a manuscript will be acknowledged by a card bearing the name of the Editor or Associate Editor who is assigned responsibility for the review process. All correspondence concerning the manuscript should be addressed to the Editor or Associate Editor. Effective January 1, 1986, all manuscripts submitted to BLOOD and subsequently accepted for publication will be assessed a $30.00 charge per published page. This charge is necessary to offset the increasing costs of publication.

Papers reporting human experimentation will be reviewed in accordance with the precepts established by the Helsinki Declaration. Copies of this declaration may be obtained by writing to the American Medical Association, 535 N Dearborn St, Chicago, IL 60610. Such papers must include a statement that the human investigations were performed after approval by a local Human Investigations Committee and in accord with an assurance filed with and approved by the Department of Health and Human Services where appropriate.

All manuscripts dealing with recombinant DNA research must include a description of the physical and biologic containment procedures practiced, in accord with the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules. The Editors of BLOOD will assume that the authors of research papers published in BLOOD are prepared to distribute to bona fide academic investigators exclusively for their own research any clone of cells or DNA used in the experiments.

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PREPARING THE MANUSCRIPT

The original and *two* complete copies of the manuscript (including letters to the Editor) must be submitted. Manuscripts must be typewritten, double or triple spaced on good quality 8½-by-11-inch white paper with margins of at least one inch. Please do not use erasable bond.

The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which work was done; (4) acknowledgments for research support; (5) name and address of the author to whom communications regarding the manuscript should be directed.

The second page should contain an abstract of 200 words or less, summarizing the reason for the study, the methods used, the results, and the major conclusions. Do not include a summary at the end of the paper.

PREPARING ILLUSTRATIONS AND TABLES

Tables and illustrations must be cited in order in the text using arabic numerals. All line drawings should be submitted as clear, glossy, black and white photographs. Hand-drawn or hand-lettered illustrations are unacceptable. Legible copies may be used only with the duplicate manuscripts. Photomicrographs and other photographic illustrations must be submitted in triplicate; photocopies of photographs and line drawings are not acceptable. The name of the first author, figure number, and designation of the top of the illustration should be marked on the back of each illustration with a soft lead pencil. Authors should avoid mounting illustrations on boards unless mounting is necessary to insure proper placement. Legends for illustrations should be typewritten, double spaced, on a separate sheet, and included at the end of the manuscript. A legend must accompany each illustration. Contributors will pay all charges involved in processing and printing of color photographs.

Figures, especially charts, graphs, and line drawings, are generally reduced in size for publication (consult a recent issue of the journal for examples). To maintain legibility it is important that all numbers, letters, and symbols be large enough originally so that *when reduced* they will remain at least 1/12 inch (2 mm) high (ie, approximately the same size as used for this line of type). Figures not properly prepared will have to be returned to the contributor for revision or will be relettered by the printer and the cost charged to the contributor.

Each table should be typed on a separate sheet and appropriately numbered. Table legends should be typed on the same sheets as the tables.

Failure to comply with the specifications for preparing illustrations and tables may result in a delay of the review process.

REFERENCES

References should be compiled at the end of the article according to the order of citation in the text. They should be typewritten, double spaced, under the heading REFERENCES. Abbreviations for titles of medical periodicals should conform to those used in the latest edition of *Index Medicus.* (A "List of Journals Indexed in Index Medicus"—with abbreviations—is obtainable from the Superintendent of Documents, US Government Printing Office, Washington, DC 20402, at a modest charge.) Name all authors. Supply only first page number for each reference. References to abstracts and letters must be identified as such.

Personal communications and references to publications in press by authors other than those submitting the paper must be substantiated by a letter from the investigator(s) concerned confirming the data or observations and granting the authors permission to cite the material.

EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

Journal article, in press:

2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

Complete book:

3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

Chapter of book:

4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): Gamma Globulins, Proceedings of the Third Nobel Symposium. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

PROOFREADING

Contributors are provided with galley proofs and are asked to proofread them for typesetting errors. Important changes in data are allowed, but authors will be charged for excessive alterations in proof. Galley proofs should be returned within 48 hours.

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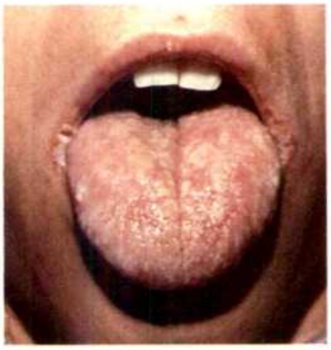
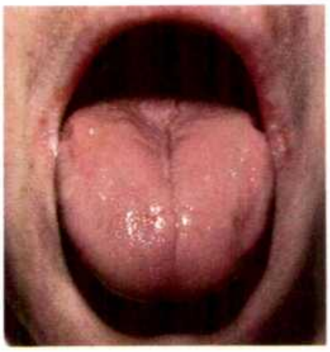
ANNOUNCEMENTS

Announcements of meetings, conferences, and the like that are of interest to the readership of BLOOD should be sent to the Editor at least three months before the first day of the month of issue. Fees for announcements vary, depending on their size and the number of issues involved. Prices may be obtained by contacting the BLOOD office.

The first troche treatment for oropharyngeal candidiasis.


Before treatment.[1]


After treatment with MYCELEX Troche.[1]

High clinical cure* rates.

A clinical cure rate of 96% was achieved in one randomized double-blind trial[2] in which MYCELEX Troche was administered to patients with oropharyngeal candidiasis. Other investigators have concluded that MYCELEX Troche "is highly effective treatment for chronic oral candidiasis."[3]

Dramatic improvement in symptoms.

Patients attain a high degree of relief at the end of therapy, as evidenced by the dramatic decrease in severity of glossitis, patches, perleche, pain, and other clinical signs and symptoms of thrush.[4]

Good patient acceptance, minimal side effects.

With MYCELEX Troche, messy suspensions and lengthy swishing are things of the past. MYCELEX Troche has a pleasant, slightly sweet taste. And an entire day's therapy—5 troches—can easily be carried in pocket or purse.

Based on a study of 52 episodes of oropharyngeal candidiasis, the investigators stated: "We believe that the troche form of clotrimazole...in the treatment of thrush in any patient population...is both effective and safe."[2†]

*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

Mycelex® Troche

(clotrimazole) 10 mg

The only troche
for oropharyngeal
candidiasis.

MYCELEX® (clotrimazole) TROCHE

Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C. Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals.

MILES
Miles Pharmaceuticals
Division of Miles Laboratories, Inc.
West Haven, Connecticut 06516 USA

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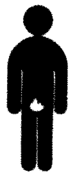
SIX WARNING SIGNS OF KIDNEY RELATED DISEASES

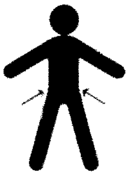


Burning or difficulty during urination

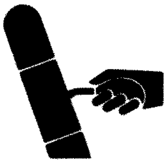
More frequent urination, particularly at night

Passage of bloody-appearing urine

Puffiness around eyes, swelling of hands and feet, especially in children



Pain in small of back just below the ribs (not aggravated by movement)



High Blood Pressure

For further information contact:

KF

National Kidney Foundation of New York, Inc.
432 Park Avenue South
New York, NY 10016

NEW

THE ONLY LIQUID IGIV

At least 98% IgG

Ready to use without reconstitution

*Confirmed in vitro inactivation of HTLV-III virus**

NEW NATIVE IGIV

GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

LIQUID NATIVE IGIV...PURE AND SIMPLE

**The Cohn-Oncley process used to produce GAMIMUNE® N has been challenged in vitro with a virus spike of 1×10^5 of HTLV-III and found to remove and/or inactivate the viral challenge.[1] In addition, GAMIMUNE® N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 reduction of HTLV-III.[1] In a separate study conducted by the Food and Drug Administration,[2] an in vitro challenge at each of six fraction steps using HTLV-III demonstrated a cumulative reduction of greater than 1×10^{15} infectious units.*

Please see following page for brief summary of prescribing information

NEW NATIVE IGIV

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 40% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

U.S. License No. 8

REFERENCES: 1. Mitra G, Wong MF, Mosen MM, McDougal JS, Levy JA: Elimination of infectious retrovirus during preparation of immunoglobulins. Submitted for publication in Transfusion. **2.** Wells MA, Wittek AE, Epstein JS, Marcus-Sekura G, Daniel S, Tankersley DL, Preston MS, Quinnan GV: Inactivation and partition of human T-cell lymphotropic virus, type III, during ethanol fractionation of plasma. Transfusion 1986; 2C(2): 210-213.

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Diffuse-Large-Cell Lymphoma

84% Complete Response
90% of Complete Responders
Disease-Free at Two Years*

MACOP-B

*Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

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*Blenoxane**

(sterile bleomycin sulfate)

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain $FI\ O_2$ at concentrations approximately that of room air (25%) during surgery and the post-operative period.
(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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POORLY DIFFERENTIATED NEOPLASMS AND TUMORS OF UNKNOWN ORIGIN

Edited by

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HERPES SIMPLEX INFECTION IN THE COMPROMISED HOST: OFTEN SERIOUS, TOO OFTEN UNSUSPECTED

Number one in a series of typical case presentations.

Esophagitis in a leukemia patient

In patients with hematologic malignancies, the visceral organ most frequently involved by herpes simplex virus (HSV) infection is the esophagus.[1] Extensive ulcerative lesions may develop on the upper two thirds of the esophagus with confluent lesions of the distal third.[2] In some cases, however, the mucosa may be grossly unremarkable even when microscopic examination shows evidence of herpetic ulcerative esophagitis.[1] Similarly, some patients may display symptoms of dysphagia and burning retrosternal pain,[3] whereas others may appear asymptomatic.[1] The diagnostic method of choice for patients with suspected herpetic esophagitis is esophagoscopy with visualization of the mucosa and procurement of cytologic and biopsy specimens from ulcerated areas.[1]

References: 1. Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **2.** Wong KK, Hirsch MS: Herpes virus infections in patients with neoplastic disease. Diagnosis and therapy. *Am J Med* 1984; 76:464-478. **3.** Lightdale CJ, Wolf DJ, Marcucci RA, et al: Herpetic esophagitis in patients with cancer: Ante mortem diagnosis by brush cytology. *Cancer* 1977;39:223-226.

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Herpetic infections may result in extensive disease and death

Immunocompromised patients are highly vulnerable to HSV infection. Such infections may interrupt therapy and even lead to death if not detected early and managed aggressively.[1] Although the initial appearance may be mild, HSV infection may progress to severe localized mucocutaneous ulcerations. The greatest threat that herpes simplex poses is an infection that disseminates from a mucocutaneous or unrecognized primary lesion and involves the lungs, liver, esophagus, or central nervous system.[1] Furthermore, herpetic ulcers frequently provide an entry for secondary bacterial and fungal pathogens.[2]

Infections may not be diagnosed correctly without virologic study

Some investigators claim that the actual incidence of serious HSV infection is probably higher than reported because "the possibility of herpetic disease is insufficiently considered and appropriate diagnostic viral studies are often not carried out."[3] One study, for example, found histocytologic evidence of visceral herpes infection in 56 patients even though herpetic involvement was not suspected in any of the patients prior to autopsy.[2]

ZOVIRAX I.V. halts viral replication and speeds healing even in debilitated patients

ZOVIRAX I.V. has been shown to be a highly effective treatment for severe mucocutaneous HSV infection in immunocompromised patients. In controlled trials, ZOVIRAX I.V. rapidly halted viral shedding — within 3 days in most patients — and significantly shortened healing time as well as duration of pain.[4,5] Furthermore, ZOVIRAX I.V. may allow patients to return home sooner and enable patients with intraoral lesions to resume normal eating habits.[6]

ZOVIRAX I.V. is well tolerated by immunocompromised patients

A multicenter collaborative trial of ZOVIRAX I.V. in immunocompromised patients reported that: "Clinical benefits were obtained without appreciable toxicity. The most common reactions were irritation of peripheral veins used for drug infusion and a low incidence of rash.... Significant renal or hematologic toxicity did not occur, even in these seriously ill patients who were commonly receiving other drugs concomitantly."[5]

Note: Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes. Please see brief summary for more information.

References: 1. Feldman S, Cox F: Viral infections and hematological malignancies. *Clin Haematol* 1976;5:311-328. **2.** Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **3.** Muller SA, Herrmann EC, Winkelmann RK: Herpes simplex infections in hematologic malignancies. *Am J Med* 1972;52:102-114. **4.** Wade JC, Newton B, McLaren C, et al: Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation. *Ann Intern Med* 1982;96:265-269 **5.** Meyers JD, Wade JC, Mitchell CD, et al: Multicenter collaborative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. *Am J Med* 1982;73(1A):229-235. **6.** Data on file, Burroughs Wellcome Co.

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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously, or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: **1.** C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** J.C. Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am. J. Med. 73(1A): 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. 33:1452-1463, 1973.

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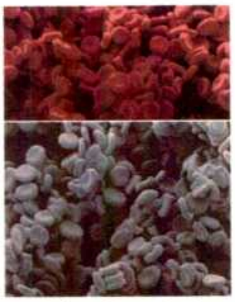

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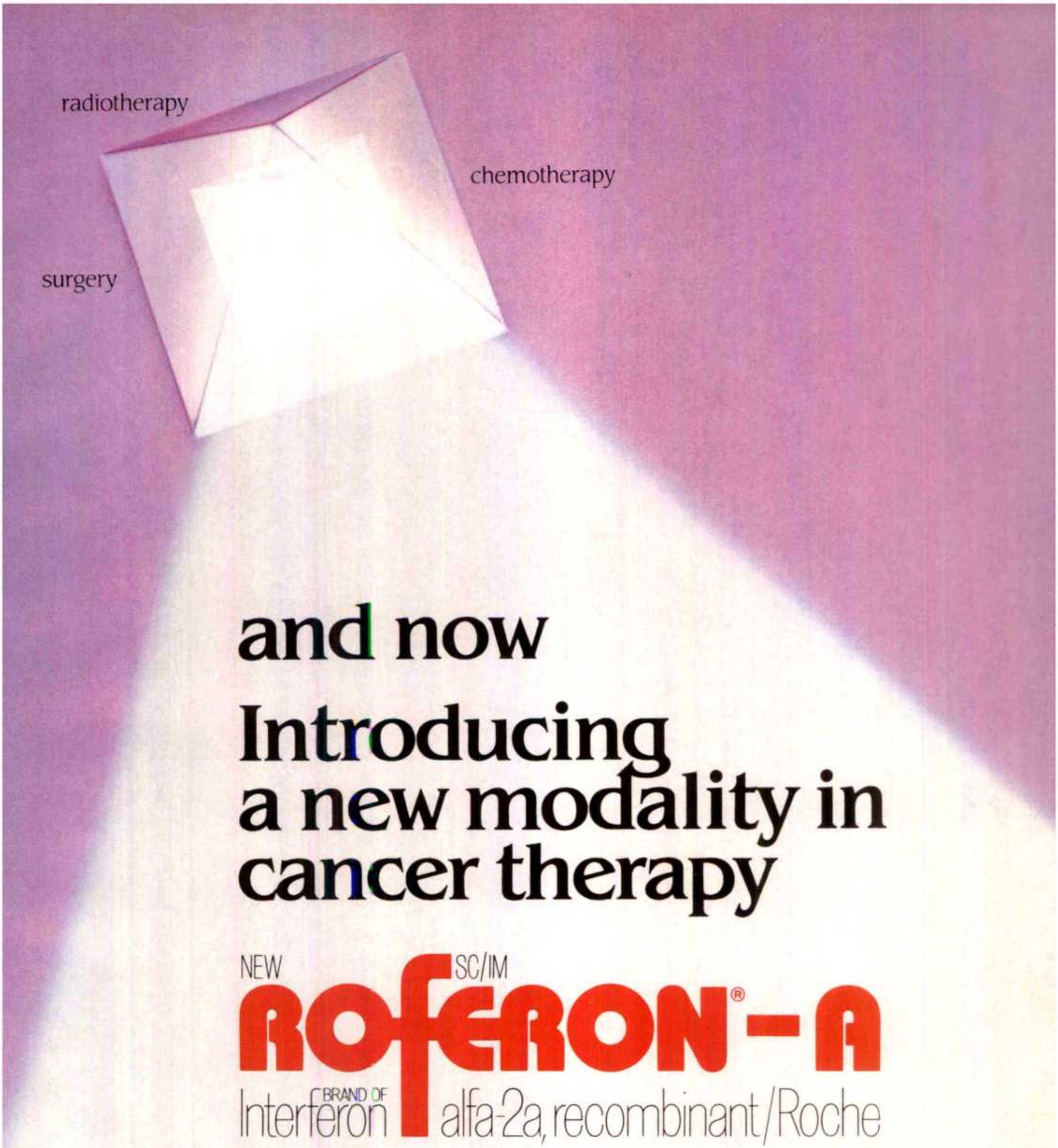

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Interferon BRAND OF alfa-2a, recombinant/Roche

Before prescribing, please see complete product information on last pages of this advertisement.

A promise fulfilled..
ROCHE
Leading the way in biotherapeutics

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first recombinant Interferon into the clinic

Vith more than a decade of dedicated esearch, Roche leads the way in idenifying, purifying and engineering the irst recombinant alfa Interferon

n 1969, scientists at the Roche Institute of Aolecular Biology began the intensive vork that culminated in the production of lfa Interferon in *E. coli* cells by means of ene-splicing and cloning technology. An dvanced technique of affinity chromatogaphy using monoclonal antibodies assures virtually pure product.

Aulticenter study included leading cancer centers...large atient data base

n 1981, ROFERON-A became the first ecombinant Interferon used in clinical rials at six leading cancer centers throughut the United States. The remarkable clinial response achieved in these trials stablished ROFERON-A as breakthrough iotherapy for hairy cell leukemia.

Study utilized fixed-dosage SC or IM injectable solution to permit self-injection by patient or administration by family member in the home

Home administration reduces the demands on busy medical staff and spares the patient time, travel "wear and tear" and expense of making frequent office or clinic visits.

Fixed-dosage SC or IM injectable solution allows administration without calculation in home, office or institution...saves time, cost-effective

Elimination of weight or body surface area calculation results in more efficient administration with less waste of time and medication. Available as ready-to-use injectable solution with no mixing or reconstitution necessary.

Common side effects usually self-limiting and manageable

Initial side effects, such as fever, chills, myalgia, headache and fatigue, are self-limiting and generally manageable. Myelosuppression, which can occur early in the treatment course, warrants close clinical and laboratory observation and is usually alleviated by dose reduction or temporary discontinuation of drug.

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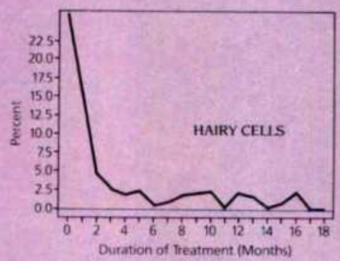
In hairy cell leukemia

The clinical response



Artist's interpretation of hairy cells based on scanning electron microscopy

With normalization of all peripheral blood counts, concomitant decrease in peripheral blood hairy cells*

ROCHE Leading the way in biotherapeutics

s remarkable

Statistically increased survival compared to a historical control group 89% improved, 11% stabilized

Treatment of hairy cell leukemia: results of multicenter study*

No. of patients evaluable for efficacy	Dosage (IU = International Units)	Response			Median Time to Response (Days)		
		Complete or Partial Remission	Minor Remission	Stabilized	Complete Remission	Partial Remission	Minor Remission
75[†]	Induction: 3 million IU SC/IM once-daily for 6 months Maintenance: 3 million IU SC/IM 3 times/week	46 **(61%)**	21 **(28%)**	8 **(11%)**	249	133	57

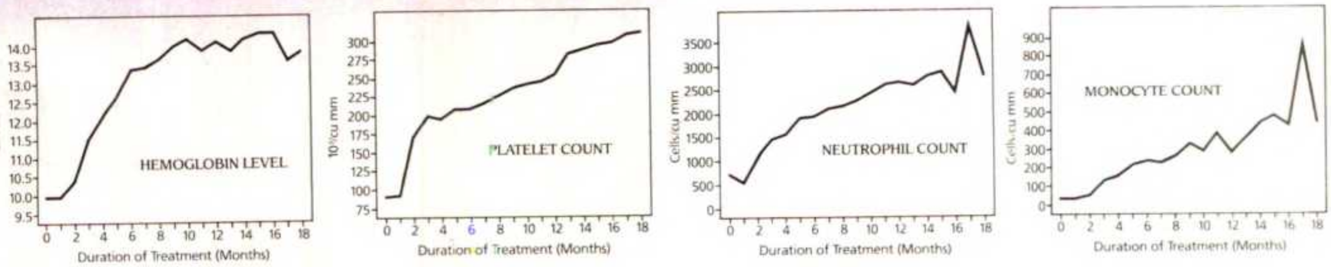
Response Criteria

	Complete	Partial	Minor
Bone Marrow Hairy Cells	0	> 50% decrease of HCL infiltrate from pretreatment values	< 50% decrease of HCL infiltrate from pretreatment values
Bone Marrow Granulocytes (sum of % of Myelocytes, Metamyelocytes and Neutrophils)	> 35%	> 35%	25-35%
Hemoglobin	> 12 Gm/dL	> 12 Gm/dL	Improvement
Absolute Neutrophil Count	> 1500 cells/cu mm	> 1500 cells/cu mm	Improvement
Platelet Count	> 100,000/cu mm	> 100,000/cu mm	Improvement

*Multicenter Study. Data on file, Hoffmann-La Roche Inc., Nutley, NJ.

[†] Of the 75, 57 had prior splenectomy and 18 had not; many were pancytopenic and transfusion-dependent; 96 patients were enrolled in study and all were evaluated for safety; 21 patients were not evaluable; 16 were not on drug long enough for efficacy evaluation at time of data analysis, 2 were removed for administrative reasons, 3 for preexisting intercurrent illness.

Normalization of blood counts*



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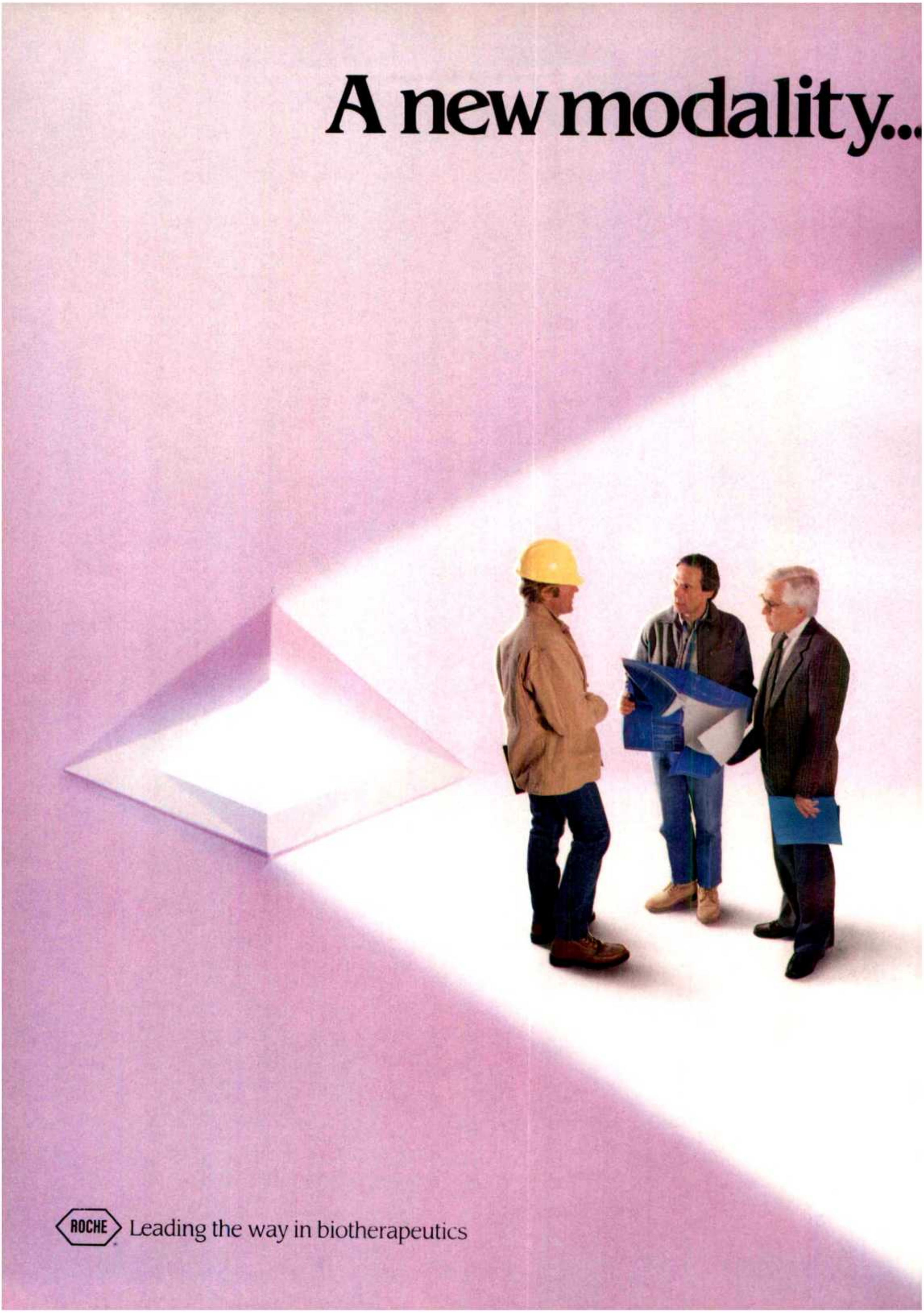

A new modality...
ROCHE
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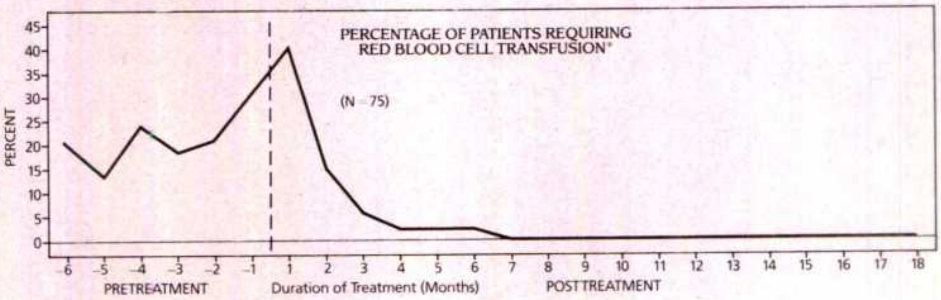
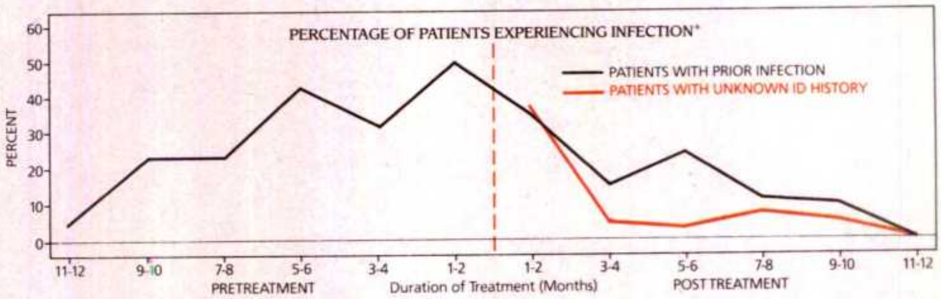
a better quality of life

From transfusion dependency to independent ambulatory treatment



From frequent life-threatening pportunistic infections to virtually none



*Multicenter Study,
Data on file,
Hoffmann-La Roche Inc.,
Nutley, NJ.

From frequent hospital visits to self-injection at home

After an initial period of hospital or clinic visits for drug administration, self-injection at home or administration by a family member makes it possible for many patients to lead a more normal life.

From the serious, difficult-to-manage side effects of chemotherapy to the more manageable side effects of biotherapy

The common side effects of ROFERON-A are rarely as debilitating as some of the serious side effects associated with standard chemotherapy and radiotherapy. (See side effects listed on following pages and in complete product information on last pages of this advertisement.)

From weight loss to weight stabilization

In a multicenter study, 86% of patients on ROFERON-A experienced no change in, or gained up to 10% of, their total body weight.

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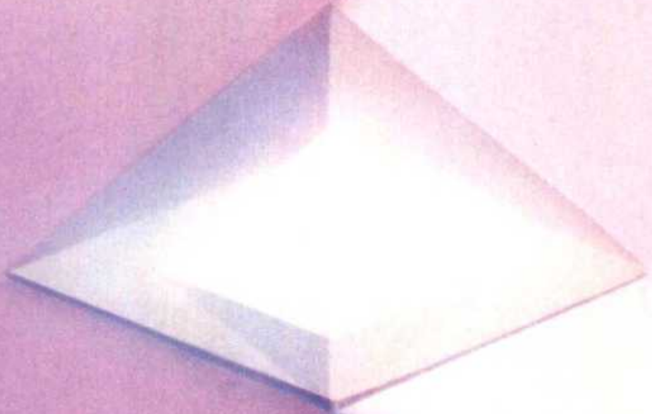
Guidelines to treatment

Patient selection

ROFERON-A (Interferon alfa-2a, recombinant/Roche) is indicated for symptomatic adult patients. It should be used with caution in patients with preexisting cardiac disease, renal and hepatic disease, myelosuppression, seizure disorders and/or compromised central nervous system function.

Information for patients

Patients should be cautioned not to change brands of Interferon without medical consultation, as a change in dosage may result. They should also be informed regarding the potential benefits and risks attendant to the use of ROFERON-A. If home use is judged desirable, instructions on appropriate use and self-injection should be given.



Patients should be well hydrated, especially during the initial stages of treatment.

Dosage and administration

Induction:	**3 million IU SC or IM once daily for 6 months.**
Maintenance:	**3 million IU SC or IM 3 times a week.**
Dosage Reduction:	**If severe reactions occur, reduce dosage by 50% or withhold individual doses.**

ROCHE Leading the way in biotherapeutics

How supplied

Ready-to-use Injectable Solution: Unit-dos vials containing 3 million IU (3 million IU/mL); multidose vials containing 18 million IU (3 million IU/0.5 mL).
No mixing or reconstitution necessary.
Must be refrigerated at temperatures of 36° to 46°F (2° to 8°C). At no time should ROFERON-A (Interferon alfa-2a, recombinant/Roche) Injectable Solution remain outside of refrigeration for more than 24 hours. Be certain all users, including physicians, nurses and patients are aware of thi important precaution. Do *not* freeze or sha

Directions for use

The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are significantly thrombocytopenic or at risk for bleeding.

Side effects: generally mild and manageable

Pretreatment

Routine history and physical examination. Determine baseline values: CBC, peripheral and bone marrow hairy cells, liver and renal function.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Flu-like symptoms

The most common initial reactions, fever, chills, myalgia, headache and fatigue, are usually self-limiting and can be ameliorated by acetaminophen.

Myelosuppressive effect

The myelosuppressive effect warrants careful monitoring for depression of blood cell

counts during initial therapy and is usually alleviated by dose reduction or temporary discontinuation of drug.

Other common but generally mild side effects

These include anorexia, nausea, vomiting, diarrhea, dizziness, rash, change in taste, dry mouth or throat, dry skin or pruritus and weight loss. Persistent mild fatigue, a problem for many patients, is usually found to be less troublesome when ROFERON-A is administered *p.m.* or *h.s.*

Rarely reported but more serious side effects

These include, but are not limited to, central nervous system effects such as decreased mental status, depression, visual disturbances, sleep disturbances and nervousness, as well as such adverse cardiac events as hypertension, chest pain, arrhythmias and palpitations.

Patient monitoring

Monitor blood parameters, liver and renal function periodically (*e.g.*, monthly) to determine if patient is responding to and tolerating drug. Response is not generally observed for one to three months. Patients usually should be treated for approximately six months before determining whether to continue therapy.

Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.

Laboratory findings

No patient had to be removed from multicenter study due to clinical laboratory abnormality.

Laboratory abnormalities, observed frequently but generally not of clinical significance, include:

SGOT elevation
  Common but not dose-limiting

Neutralizing antibodies
  Found in four out of 51 (7.8%) patients in multicenter study evaluable for the development of antibodies; no clinical sequelae or adverse effect on response, duration of response or survival has been noted thus far.

NOTE: Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.

Contraindications

ROFERON-A is contraindicated in patients with known hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

Use in fertile women

Fertile women should use effective contraception while receiving ROFERON-A.

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ROFERON®-A

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Interferon alfa-2a recombinant/Roche

DESCRIPTION: Roferon®-A (Interferon alfa-2a, recombinant/Roche) is a sterile protein product for use by injection. Roferon-A is manufactured by recombinant DNA technology that employs a genetically engineered *E. coli* bacterium containing DNA that codes for the human protein. Interferon alfa-2a, recombinant/Roche is a highly purified protein containing 165 amino acids, and it has an approximate molecular weight of 19,000 daltons. The purification procedure includes affinity chromatography using a murine monoclonal antibody. Roferon-A is supplied as an injectable solution. The solution is colorless and contains Interferon alfa-2a, recombinant/Roche, phenol as a preservative, sodium chloride for isotonicity and human serum albumin as a stabilizer. Fermentation is carried out in a defined nutrient medium containing the antibiotic tetracycline hydrochloride, 5 mg/liter. However, the presence of the antibiotic is not detectable in the final product.

Each vial of Roferon-A contains 3 million or 18 million international units (IU) of Interferon alfa-2a, recombinant/Roche. The specific activity of Interferon alfa-2a, recombinant/Roche, is 2×10^8 IU/mg protein. The route of administration is subcutaneous or intramuscular.

CLINICAL PHARMACOLOGY: The mechanism by which Interferon alfa-2a, recombinant/Roche, or any other interferon, exerts antitumor activity is not clearly understood. However, it is believed that direct antiproliferative action against tumor cells and modulation of the host immune response play important roles in the antitumor activity.

The biological activities of Interferon alfa-2a, recombinant/Roche are species-restricted, *i.e.*, they are expressed in a very limited number of species other than humans. As a consequence, preclinical evaluation of Interferon alfa-2a, recombinant/Roche has involved *in vitro* experiments with human cells and some *in vivo* experiments.[1] Using human cells in culture, Interferon alfa-2a, recombinant/Roche has been shown to have antiproliferative and immunomodulatory activities that are very similar to those of the mixture of interferon alfa subtypes produced by human leukocytes. *In vivo*, Interferon alfa-2a, recombinant/Roche has been shown to inhibit the growth of several human tumors growing in immunocompromised (nude) mice. Because of its species-restricted activity, it has not been possible to demonstrate antitumor activity in immunologically intact syngeneic tumor model systems, where effects on the host immune system would be observable. However, such antitumor activity has been repeatedly demonstrated with, for example, mouse interferon-alfa in transplantable mouse tumor systems. The clinical significance of these findings is unknown.

The metabolism of Interferon alfa-2a, recombinant/Roche is consistent with that of alfa interferons in general. Alfa interferons are totally filtered through the glomeruli and undergo rapid proteolytic degradation during tubular reabsorption, rendering a negligible reappearance of intact alfa interferon in the systemic circulation. Small amounts of radiolabeled Interferon alfa-2a, recombinant/Roche appear in the urine of isolated rat kidneys, suggesting near complete reabsorption of Interferon alfa-2a, recombinant/Roche catabolites. Liver metabolism and subsequent biliary excretion are considered minor pathways of elimination for alfa interferons.

The serum concentrations of Interferon alfa-2a, recombinant/Roche reflected a large intersubject variation in both healthy volunteers and patients with disseminated cancer.

In healthy people, Interferon alfa-2a, recombinant/Roche exhibited an elimination half-life of 3.7 to 8.5 hours (mean 5.1 hours), volume of distribution at steady-state of 0.223 to 0.748 L/kg (mean 0.400 L/kg) and a total body clearance of 2.14 to 3.62 mL/min/kg (mean 2.79 mL/min/kg) after a 36 million IU (2.2×10^6 pg) intravenous infusion. After intramuscular and subcutaneous administrations of 36 million IU, peak serum concentrations ranged from 1500 to 2580 pg/mL (mean 2020 pg/mL) at a mean time to peak of 3.8 hours and from 1250 to 2320 pg/mL (mean 1730 pg/mL) at a mean time to peak of 7.3 hours, respectively. The apparent fraction of the dose absorbed after intramuscular injection was greater than 80%.

The pharmacokinetics of Interferon alfa-2a, recombinant/Roche after single intramuscular doses to patients with disseminated cancer were similar to those found in healthy volunteers. Dose proportional increases in serum concentrations were observed after single doses up to 198 million IU. There were no changes in the distribution or elimination of Interferon alfa-2a, recombinant/Roche during twice daily (0.5 to 36 million IU), once daily (1 to 54 million IU), or three times weekly (1 to 136 million IU) dosing regimens up to 28 days of dosing. Multiple intramuscular doses of Interferon alfa-2a, recombinant/Roche resulted in an accumulation of 2 to 4 times the single dose serum concentrations. Pharmacokinetic information in patients with hairy cell leukemia is presently unknown.

The acute parenteral toxicity of Interferon alfa-2a, recombinant/Roche has been studied in mice, rats, rabbits and ferrets at doses up to 30 million IU/kg intravenously, and 500 million IU/kg intramuscularly. No treatment-related mortality was noted in any species given Interferon alfa-2a, recombinant/Roche by any of the routes of administration.

Effects on Hairy Cell Leukemia: During the first one to two months of treatment of patients with hairy cell leukemia, significant depression of hematopoiesis was likely to occur. Subsequently, there was improvement in circulating blood cell counts.

Of the 75 patients who were evaluable for efficacy following at least 16 weeks of therapy, 46 (61%) achieved complete or partial response. Twenty-one patients (28%) had a minor remission, eight (11%) remained stable, and none had worsening of disease. All patients who achieved either a complete or partial response had complete or partial normalization of all peripheral blood elements including hemoglobin level, white blood cell, neutrophil, monocyte and platelet counts with a concomitant decrease in peripheral blood and bone marrow hairy cells. Responding patients also exhibited a marked reduction in red blood cell and platelet transfusion requirements, a decrease in infectious episodes and improvement in performance status. The probability of survival for two years in patients receiving Roferon-A (94%) was statistically increased compared to a historical control group (75%).

ROFERON®-A (Interferon alfa-2a, recombinant/Roche)

INDICATIONS AND USAGE: Roferon-A is indicated for use in the treatment of hairy cell leukemia in people 18 years of age or older. Studies have shown that Roferon-A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and nonsplenectomized patients.[2,3]

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically (*e.g.*, monthly) during treatment to determine whether response to treatment has occurred. If a patient does not respond within six months, treatment should be discontinued. If a response to treatment does occur, treatment should be continued until no further improvement is observed and these laboratory parameters have been stable for about three months. It is not known whether continued treatment after that time is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS: Roferon-A is contraindicated in patients with known hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

WARNINGS: Roferon-A should be administered under the guidance of a qualified physician. (See DOSAGE AND ADMINISTRATION.) Appropriate management of the therapy and its complications is possible only when adequate diagnostic and treatment facilities are readily available.

Roferon-A should be used with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised central nervous system function.

Because of the possibility of severe or even fatal adverse reactions, patients should be informed not only of the benefits of therapy but also of the risks involved.

Roferon-A should be administered with caution to patients with cardiac disease or with any history of cardiac illness. No direct cardiotoxic effect has been demonstrated, but it is likely that acute, self-limited toxicities (*i.e.*, fever, chills) frequently associated with Roferon-A administration may exacerbate preexisting cardiac conditions. Rarely, myocardial infarction has occurred in patients receiving Roferon-A.

Caution should be exercised when administering Roferon-A to patients with myelosuppression.

Central nervous system adverse reactions have been reported in a number of patients. These reactions included decreased mental status, exaggerated central nervous system function, and dizziness. More severe obtundation and coma have been rarely observed. Most of these abnormalities were mild and reversible within a few days to three weeks upon dose reduction or discontinuation of Roferon-A therapy. Careful periodic neuropsychiatric monitoring of all patients is recommended.

Leukopenia and elevation of hepatic enzymes occurred frequently but were rarely dose-limiting. Thrombocytopenia occurred less frequently. Proteinuria and increased cells in urinary sediment were also seen infrequently. Rarely, significant hepatic, renal and myelosuppressive toxicities were noted.

PRECAUTIONS: *General:* In all instances where the use of Roferon-A is considered for chemotherapy, the physician must evaluate the need and usefulness of the drug against the risk of adverse reactions. Most adverse reactions are reversible if detected early. If severe reactions occur, the drug should be reduced in dosage or discontinued and appropriate corrective measures should be taken according to the clinical judgment of the physician. Reinstitution of Roferon-A therapy should be carried out with caution and with adequate consideration of the further need for the drug and alertness as to possible recurrence of toxicity.

The minimum effective dose of Roferon-A for treatment of hairy cell leukemia has not been established.

Information for Patient: Patients should be cautioned not to change brands of Interferon without medical consultation, as a change in dosage may result. Patients should be informed regarding the potential benefits and risks attendant to the use of Roferon-A. If home use is determined to be desirable by the physician, instructions on appropriate use should be given, including review of the contents of the enclosed Patient Information Sheet. Patients should be well hydrated, especially during the initial stages of treatment.

Laboratory Tests: Periodic complete blood counts and liver function tests should be performed during the course of Roferon-A treatment. They should be performed prior to therapy and at appropriate periods during therapy. Since responses of hairy cell leukemia are not generally observed for one to three months after initiation of treatment, very careful monitoring for severe depression of blood cell counts is warranted during the initial phase of treatment.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis and Impairment of Fertility.

Carcinogenesis: Roferon-A has not been tested for its carcinogenic potential.

Mutagenesis: A. Internal studies—Ames tests using six different tester strains, with and without metabolic activation, were performed with Roferon-A up to a concentration of 1920 µg/plate. There was no evidence of mutagenicity.

Human lymphocyte cultures were treated *in vitro* with Roferon-A at noncytotoxic concentrations. No increase in the incidence of chromosomal damage was noted.

B. Published studies—There are no published studies on the mutagenic potential of Roferon-A. However, a number of studies on the genotoxicity of human leukocyte interferon have been reported.

A chromosomal defect following the addition of human leukocyte interferon to lymphocyte cultures from a patient suffering from a lymphoproliferative disorder has been reported.

In contrast, other studies have failed to detect chromosomal abnormalities following treatment of lymphocyte cultures from healthy volunteers with human leukocyte interferon.

It has also been shown that human leukocyte interferon protects primary chick embryo fibroblasts from chromosomal aberrations produced by gamma rays.

Impairment of Fertility. Roferon-A has been studied for its effect on fertility in Macaca mulatta (rhesus monkeys). Nonpregnant rhesus females treated with Roferon-A at doses of 5 and 25 million IU/kg/day have shown menstrual cycle irregularities, including prolonged or shortened menstrual periods and erratic bleeding; these cycles were considered to be anovulatory. These monkeys returned to a normal menstrual rhythm following discontinuation of treatment.

Drug Interactions: Interactions between Roferon-A and other drugs have not been fully evaluated.

OFERON®-A (Interferon alfa-2a, recombinant/Roche)

REGNANCY: *Teratogenic Effects:* Pregnancy Category C. Roferon-A has een shown to demonstrate a statistically significant increase in abortifacient tivity in rhesus monkeys when given at approximately 20 to 500 times the man dose. A study in pregnant rhesus monkeys treated with 1, 5 or 25 illion IU/kg/day of Roferon-A in their early to midfetal period (days 22 to 70 gestation) has failed to demonstrate teratogenic activity for Roferon-A. here are no adequate and well-controlled studies in pregnant women. *onteratogenic Effects:* Dose-related abortifacient activity was observed in egnant rhesus monkeys treated with 1, 5 or 25 million IU/kg/day of oferon-A in their early to midfetal period (days 22 to 70 of gestation). A te-fetal period study (days 79 to 100 of gestation) is in progress and as yet ere have been no reports of any increased rate of abortion. *sage in Pregnancy:* Safe use in human pregnancy has not been established. Therefore, Roferon-A should be used during pregnancy only if the tential benefit justifies the potential risk to the fetus. Information from imate studies showed dose-related menstrual irregularities and an creased incidence of spontaneous abortions. Therefore, fertile women ould not receive Roferon-A unless they are using effective contraception ring the therapy period.

ale fertility and teratologic evaluations have yielded no significant adverse fects to date.

ursing Mothers: It is not known whether this drug is excreted in human ilk. Because many drugs are excreted in human milk and because of the tential for serious adverse reactions in nursing infants from Roferon-A, a ecision should be made whether to discontinue nursing or to discontinue the ug, taking into account the importance of the drug to the mother.

ediatric Use: Safety and effectiveness in children under 18 years of age ave not been established.

DVERSE REACTIONS: The following data on adverse reactions are ased on the subcutaneous or intramuscular administration of Roferon-A as single agent for the treatment of 63 patients with hairy cell leukemia during vestigational trials in the United States.

lu-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), yalgias (73%) and headache (71%) occurred in the majority of patients and nded to diminish with continuing therapy. Other side effects such as norexia (46%), nausea (32%), emesis (10%), diarrhea (29%), dizziness 21%), rash (18%), change in taste (13%), dryness or inflammation of the ropharynx (16%), dry skin or pruritus (13%) and weight loss (14%) were bserved with moderate frequency. Less commonly, diaphoresis (8%), aresthesias (6%), numbness (6%), partial alopecia (8%), reactivation of erpes labialis (8%), transient impotence (6%) and arthralgias (5%) were lso observed. Rarely (<3%), central nervous system effects including ecreased mental status, depression, visual disturbances, sleep disturances and nervousness, as well as cardiac adverse events, including ypertension, chest pain, arrhythmias and palpitations, were reported. dverse experiences which occurred rarely and may have been related to nderlying disease, included epistaxis, bleeding gums, ecchymosis and etechiae. Miscellaneous adverse events, such as night sweats, urticaria, onjunctivitis and inflammation at the site of injection, were also rarely bserved.

oferon-A has also been evaluated for the treatment of many other types of ancer under investigational trials in the United States. These studies enerally utilized higher doses (12 to 50 million IU/m²). All of the previously escribed adverse reactions which occurred in patients with hairy cell ukemia were also observed in patients receiving higher doses. The incience of most adverse reactions was similar between the two groups, but nded to be more severe in patients who received higher doses of oferon-A. Some additional adverse effects which occurred in these patients cluded confusion (10%), hypotension (6%), lethargy (3%) and edema 3%). Adverse experiences occurring in less than 1% of these patients and bserved only in patients with malignancies other than hairy cell leukemia re as follows: *Gastrointestinal*—abdominal fullness, hypermotility and epatitis; *Central Nervous System*—gait disturbance, poor coordination, halcinations, syncope, seizures, encephalopathy, psychomotor retardation, oma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, ysphasia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional labily, irritability, hyperactivity, involuntary movements, claustrophobia and loss f libido; *Peripheral Nervous System*—muscle contractions; *Cardiovascular*—congestive heart failure, pulmonary edema, myocardial infarction, aynaud's phenomenon and hot flashes; *Pulmonary*—bronchospasm and achypnea; *Miscellaneous*—excessive salivation and flushing of skin. *bnormal Laboratory Test Values:* The percentage of patients with hairy cell eukemia or with other types of malignancies who experienced a significant bnormal laboratory test value at least once during their treatment is shown n the following table:

ABNORMAL LABORATORY TEST VALUES

	Hairy Cell Leukemia (n = 63)	Overall Safety Summary* (n = 1019)
ematologic		
eukopenia	59%	69%
eutropenia	39%	58%
hrombocytopenia	42%	42%
ecreased Hemoglobin	36%	6.3%
ecreased Hematocrit	43%	12.5%
ean Time to Nadir		
WBC	38 days	22 days
Platelets	19 days	17 days
epatic		
GOT	47%	78%
lkaline Phosphatase	18%	48%
DH	12%	47%
ilirubin	1.6%	31%
enal/Urinary		
UN	2%	10%
erum Creatinine	3%	10%
ric Acid†	6%	15%
roteinuria†	10%	25%
ther Chemistry Tests		
ypocalcemia	10%	51%
levated FBS‡	33%	39%
levated Serum Phosphorus	2%	17%

ROFERON®-A (Interferon alfa-2a, recombinant/Roche)

*Patients with hairy cell leukemia are included in this overall summary.

†The majority of abnormal uric acid values and proteinuria were of minimal severity. Since these abnormalities are commonly observed in patients with advanced malignancy, it is difficult to ascertain their relationship to Roferon-A treatment.

‡Random glucose determination.

Neutralizing antibodies to Roferon-A were detected in approximately 27% of all patients (3.4% for patients with hairy cell leukemia). No clinical sequelae of their presence have been documented thus far. Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.[4]

DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The induction dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as a subcutaneous or intramuscular injection. Subcutaneous administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count <50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, three times per week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended.

Patients should be treated for approximately six months before the physician determines whether to continue therapy in patients who respond or discontinue therapy in patients who did not respond. Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.

If severe reactions occur, dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve. The minimum effective dose of Roferon-A has not been established.

DIRECTIONS FOR USE: The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are thrombocytopenic (platelet count <50,000) or who are at risk for bleeding.

Injectable Solution:

3 million IU Roferon-A per vial—Each 1 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative.

18 million IU Roferon-A per vial (for multiple-dose use)—Each 1 mL contains 6 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative. Each 0.5 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche.

The injectable solution should be stored in the refrigerator at 36° to 46°F (2° to 8°C). Do *not* freeze. Do not shake.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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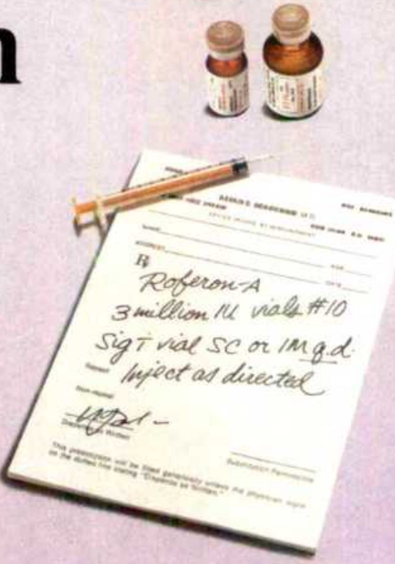

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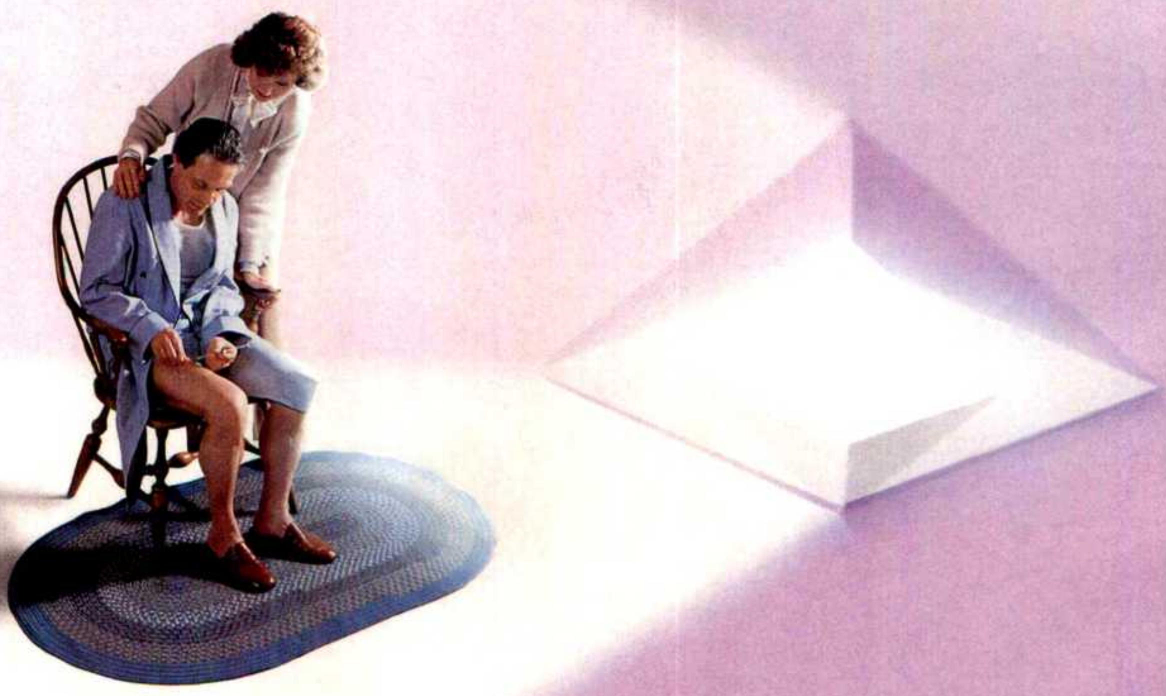

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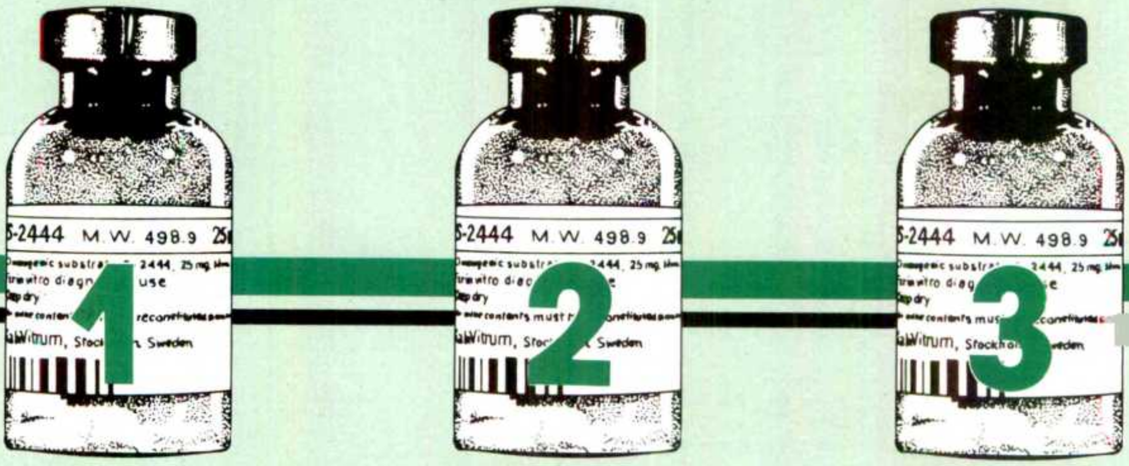

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DESCRIPTION: Each 1 ml ampul of Leucovorin Calcium *Lederle* contains Leucovorin 3 mg as the calcium salt which is the form preferred for intramuscular injection Preservative benzyl alcohol 0 9% w/v The inactive ingredients are sodium chloride 0 56% w/v. Water for Injection qs 100% and sodium hydroxide or hydrochloric acid is used to adjust the pH to approximately 7 7

Each 50 mg vial of Leucovorin Calcium cryodesiccated powder when reconstituted with 5 ml of sterile diluent contains Leucovorin 10 mg per ml as the calcium salt which is the form preferred for intramuscular injection Contains no preservative Dilute only with Bacteriostatic Water for Injection USP which contains benzyl alcohol The inactive ingredients are sodium chloride 40 mg/vial, and sodium hydroxide or hydrochloric acid qs to pH approximately 8 1 When reconstituted as directed, the resulting solution must be used within seven days If the product is reconstituted with Water for Injection USP, use immediately

ACTIONS: Leucovorin (folinic acid) is the formyl derivative and active form of folic acid

Leucovorin Calcium is useful clinically in circumventing the action of folate reductase There is no evidence that intramuscular doses of greater than 1 mg have greater efficacy than those of 1 mg

INDICATIONS: Indicated (a) to diminish the toxicity and counteract the effect of inadvertently administered overdosages of folic acid antagonists (See Warnings) (b) In the treatment of the megaloblastic anemias due to sprue, nutritional deficiency, pregnancy, and infancy when oral therapy is not feasible

CONTRAINDICATIONS: Not to be administered for the treatment of pernicious anemia or other megaloblastic anemias where Vitamin B_{12} is deficient

WARNINGS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to lack of Vitamin B_{12} A hematologic remission may occur while neurologic manifestations remain progressive

In the treatment of overdosage of folic acid antagonists Leucovorin must be administered within one hour, if possible, and is usually ineffective if administered after a delay of four hours

PRECAUTIONS: In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid

DOSAGE: Megaloblastic anemia No more than or up to 1 mg daily There is no evidence that intramuscular doses greater than 1 mg daily have greater efficacy than those of 1 mg, additionally, loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg

For the treatment of overdosage of folic acid antagonists To be given in amounts equal to the weight of the antagonist given

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Rev 11/80

LEUCOVORIN CALCIUM TABLETS

BRIEF SUMMARY

Before prescribing Leucovorin Calcium Tablets, please consult complete prescribing information

INDICATIONS AND USAGE: Leucovorin Calcium Tablets are indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS)

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12} A hematologic remission may occur while neurologic manifestations remain progressive

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible As the time interval between antifolate administration (eg, methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes

PRECAUTIONS: *General* Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin In the presence of pernicious anemia, a hematologic remission may occur while neurologic manifestations remain progressive Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney

Drug Interactions Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin, and primidone, and increase the frequency of seizures in susceptible children

Pregnancy Teratogenic Effects Pregnancy Category C Animal reproduction studies have not been conducted with leucovorin It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity Leucovorin should be given to a pregnant woman only if clearly needed

Nursing Mothers It is not known whether this drug is excreted in human milk Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother

Pediatric Use (See Drug Interactions)

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase Leucovorin rescue must begin within 24 hours of antifolate administration A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for 72 hours If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the premethotrexate serum creatinine, the leucovorin dosage should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M [7,8] The recommended dosage of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (ie, trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators [9,10,11]

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VOL 68, No 2

AUGUST 1986

REVIEW

The Structure of the T Cell Antigen Receptor Genes in Normal and Malignant T Cells

By Mark D. Minden and Tak W. Mak

T CELLS play an essential role in specific immune responses. To perform this function, they must be able to recognize a wide range of foreign antigens. Unlike immunoglobulins which recognize free antigens, T cells recognize foreign antigens in the context of their own cells' major histocompatibility complex (MHC) gene products, a phenomenon known as MHC restriction.[1] Helper and T4 cytotoxic T cells interact with antigens in the context of Class II MHC gene products, while T8 cytotoxic T cells recognize antigens in conjunction with Class I MHC gene products. For many years, the molecular structure of the T cell antigen receptor and the manner by which this structure can recognize antigen was hotly debated.[2,3] Some investigators felt that T cells use segments of the immunoglobulin genes[4,5] while others postulated that separate genes were used.[6,7] Recently, as a result of the cloning of the T cell antigen receptor (TcR) genes,[8,9] answers to these questions are available.[8-10] In this review, we will describe the structure of TcR genes in normal and malignant T cells. The use of these genes in the classification and the management of patients with T cell malignancies and other hyperplastic disorders of the lymph node as well as in studies of chromosomal translocation involving T cell malignancies will also be discussed.

Cloning of the Human T Cell Antigen Receptors

The use of monoclonal antibodies raised against individual clones of T cells provided evidence for a distinct antigen-specific structure on T cells.[11-14] These monoclonal antibodies, designated anticlonotypic antibodies, were raised against a given T cell clone specific for one antigen and then shown to be able to differentiate this clone, from T cell clones specific for another antigen. These antibodies were used to immunoprecipitate structures of approximately 90 kilodaltons (kd) from the surface of T cells. Under reducing conditions, this structure separated into two components of approximately 50 kd and 40 kd from human T cells. The larger and more acidic glycoprotein was termed the α chain, while the smaller and more basic polypeptide was termed the β chain.

Using molecular biology to approach the study of this receptor, two groups isolated cDNA clones that coded for the β chain of the TcR.[8,9] They first used subtractive hybridization and differential screening to identify a number of cDNA clones that appeared only in T cells and not in B cells. A small proportion of these clones were shown to undergo somatic rearrangements in clones of functional T cells and leukemic cell lines of thymic origin.[9,15] The primary structure of the proteins deduced from the cDNAs' sequence revealed significant homology with the products of the immunoglobulin and MHC genes.[8,16,17] Through comparison with partial protein sequences of the β chain these cDNA clones were identified as the β chain of the TcR. The α chain of the TcR was isolated subsequently using similar techniques or by the use of oligonucleotide probes deduced from partial protein sequences of the α chain.[17-21] A third T cell–specific gene that undergoes somatic rearrangement during T cell development has also been cloned. This gene, designated the γ chain, has no known function at the present time.[22-24]

Protein Structures of the α and β Chain

The primary structure of the α and β chain proteins was deduced from the DNA sequence of their cDNAs.[8,21,25] Both proteins are composed of a variable (V) and a constant (C) domain connected by a diversity (D) (at least for β chain) and a joining (J) segment. The overall structure of each chain is very similar to that of a light chain immunoglobulin molecule, with an extended transmembrane and cytoplasmic portion. These two chains are most likely held together by disulphide bonds located just above the transmembrane region. The similarity of their primary protein structures to immunoglobulin and MHC products suggests that these genes may have evolved from a common primordial precursor and that these structures have been retained to maintain mutual interactions of their protein products during an immune response reaction.[10]

From the Ontario Cancer Institute, Toronto, Canada.

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Genomic Organization of the α and β Chain Genes of the T-Cell Antigen Receptor

When the cDNA of α or β chain was used to probe germline genomic DNA from fibroblasts, multiple bands were observed.[8,15,26] DNA from cloned T cells, leukemic T cells, or leukemic T cell lines also showed multiple bands although the patterns of the bands were different from those of fibroblasts (germline pattern) and of each other. These differences were not the result of an inherited restriction fragment length polymorphism as DNA from leukemic T cells was, in a number of cases, compared to DNA from fibroblasts derived from the same patient.[26] This finding, that the structure of the DNA in individual T cell clones and leukemic T cells has a different organization from fibroblast DNA and from each other, indicates that different somatic rearrangements of TcR DNA have occurred in different T cell clones (Fig 1).

Organization of the β chain genes. The organization of the variable, diversity, joining, and constant region of the β gene TcR has now been largely elucidated (Fig 2). These genes are located on chromosome 7q in man and 6 in mouse.[27,28] At the 5′ end of the variable region segments is a leader sequence separated from the rest of the coding sequence by a single intron. Located immediately 3′ of these coding sequences are structures consisting of highly conserved heptamer and nonamer sequences separated by a nonconserved spacer.[29] Based on Southern blot analysis and sequences of variable regions derived from cDNAs, it appears that there are approximately 50 variable region genes from over 12 related families.[30] The number of V genes is considerably smaller than the number of kappa and immunoglobulin heavy chain V genes.[10] Comparison of the RNA sequences to the genomic germline sequences indicates that somatic point mutation is limited and thus may play only a minor role in the generation of diversity of these T cell genes.[31]

The diversity, joining segments, and constant region genes are located in a region of approximately 20 kb.[32] A restriction enzyme map and the general layout of these genes are shown in Fig 2. Two highly conserved constant region genes can be found approximately 10 kb apart. The 5′ C region has been designated $C_{\beta 1}$, while the 3′ C region has been referred to as $C_{\beta 2}$. These two genes are not only highly homologous to each other, but their general organization is also very similar. Their first two exons encode most of the extracellular constant domain, the third exon contains sequences corresponding to part of the transmembrane region, while the last exon contains sequences encoding the cytoplasmic domain, as well as the 3′ untranslated region of the β chain message. While the coding sequences of these two C regions are highly homologous, the 3′ noncoding regions, as well as the introns, exhibit little similarity to each other. The lack of homology between the 3′ nontranslated sequences has been used to distinguish transcripts from the two C regions.[33] Approximately 4 kb 5′ of each of the C regions is a cluster of joining segments. In the human, these clusters are composed of 6 and 7 J segments upstream of $C_{\beta 1}$ and $C_{\beta 2}$, respectively.[32] Adjacent to the 5′ end of each of the J segments are the highly conserved nonamer and heptamer structures (separated by a spacer). Like the immunoglobulin heavy chain genes, TcR β chain genes contain diversity segments. There is a single D segment located approximately 650 basepairs 5′ of each J cluster.[32,34] Each D segment is flanked on the 5′ and 3′ side by a heptamer and a nonamer structure which is involved in DNA rearrangement. The similarity in the overall organization of the two constant region genes and the associated D and J segments has led to the proposal that this tandem structure arose through gene duplication.[10,32]

Organization of the α chain T cell receptor genes. The α

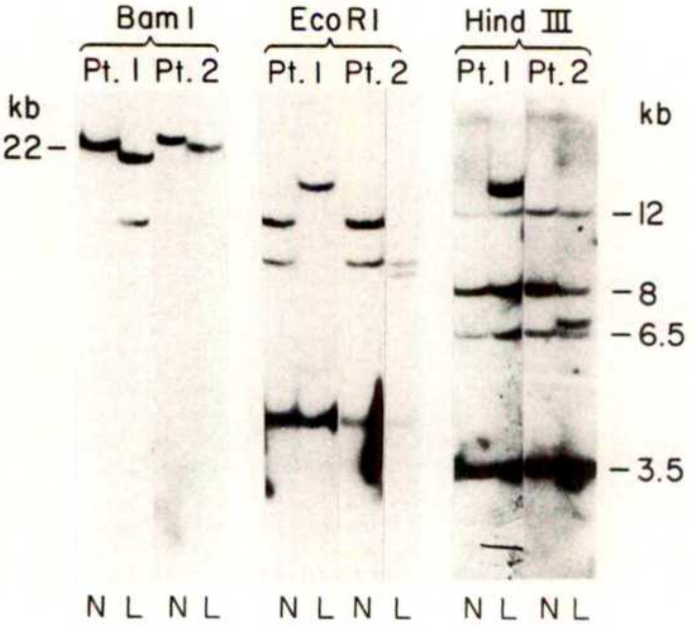

Fig 1. A Southern blot of DNA extracted from the fibroblasts and leukemic cells of two different patients. The DNA was cut with either Bam HI, Eco RI, or Hind III separated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with a constant region probe for TcR β. Comparing the leukemic DNA to the fibroblast DNA reveals that in both cases and with all three enzymes rearrangement of TcR is evident. The 9.5 kb Eco RI band present in this blot and Fig 6 is due to contamination of the probe with V and J sequences and is not the result of partial digestion.

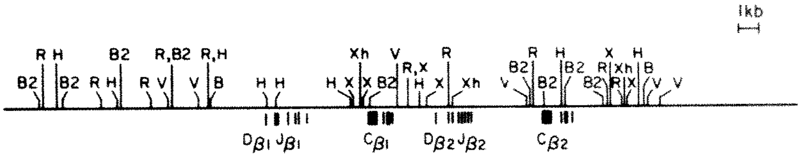

Fig 2. A restriction enzyme map of the constant region of TcR β. The restriction enzymes used are Bam HI (B), BglII (B2), Eco RI (R), Eco RV (V), Hind III (H), XbaI (X), and XhoI (Xh).

chain gene is also organized in a manner similar to that of immunoglobulin.[17,21] Unlike the β chain genes, the α chain genes in man and mouse contain only one constant region. These genes have been mapped to chromosome 14 in man in the region of 14q12 with the variable region being proximal and the constant region distal, with respect to the centromere.[35-38] Genomic organization of the α chain V regions is also very similar to those of the β chain and immunoglobulin genes. The V_α genes contain an intron that divides leader sequences from the rest of the coding sequences. An initial estimation suggests that the α chain gene probably has on the order of 50 V region segments.[39] The constant region is divided into four exons with the first two encoding the extracellular domain, while the third contains sequences that code for the transmembrane and cytoplasmic portion of the protein. Unlike the immunoglobulin and β chain constant regions, the 3′ untranslated region of this gene occupies its own exon.[25]

Perhaps the most unusual feature of the α chain genes are the J gene segments.[25] These J genes have been found to occupy a region greater than 50 kb 5′ of the C region (unpublished result). The number of these J gene segments is still unknown although there appears to be more than 20 individual germline segments. A schematic diagram and restriction enzyme map of this region in man is illustrated in Fig 3. Although a diversity segment has not been identified, comparison of α chain cDNAs indicates that such structures likely exist. Preliminary studies have found that point mutation in coding regions of α and β chain genes are unusual. This would indicate that T cell receptor diversity arises largely from recombinatorial joining between the V, D, and J segments as well as N-region diversity.[40,41]

Recombination of T-Cell Receptor Genes

The somatic recombination events leading to the joining of the V, D, and J segments of the T cell receptor α and β chain genes are probably very similar to those involving immunoglobulin (Ig) genes.[40,41] This assumption is based on the findings that recombinational signals found adjacent to the V, D, and J segments of TcR are very similar to those found in the Ig genes. The use of a similar mechanism may explain the finding that in some T cell lines the Ig heavy chain genes are rearranged,[42] and in some B-cell lines TcR β gene is rearranged.[43] This observation is not restricted to cell lines but has also been seen in some leukemias.[44] Similar to their Ig counterparts, the first event in rearrangement is probably a DJ joining.[45-47] This rearrangement can sometimes lead to the expression of a truncated transcript of approximately 1 kb for β chain[21,30] and 1.3 kb for α chain[21] that contains only D, J, and C sequences together with sequences 5′ of the D segment or a transcript of 1 kb that contains J, C, and 5′ J germline sequences from an unrearranged gene. These transcripts can be found occasionally in B cells.[21] A second and final recombination event brings the V region genes adjacent to the DJ segments. This rearrangement leads to the expression of a fully mature 1.3 or 1.6 kb message from the β[33] and α chain genes,[21] respectively (Figs 4 and 5).

Expression of a Functional T-Cell Antigen Receptor on the Cell Surface

As the TcR is responsible for activating T lymphocytes when they encounter antigens, TcR must be present on the cell surface, and by itself or in association with other proteins must be able to transmit controlling signals through the

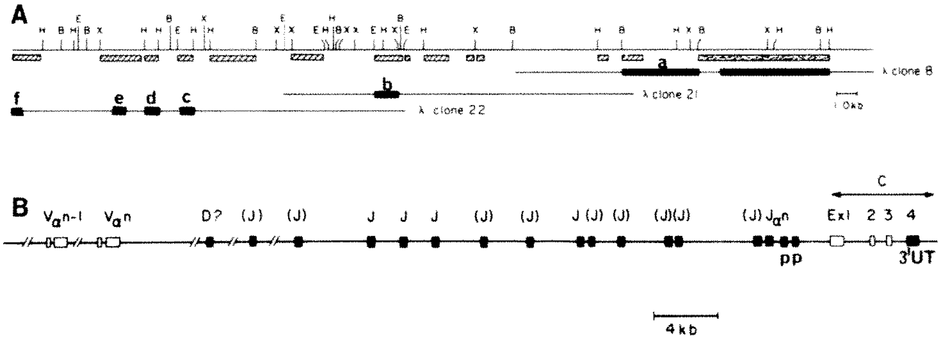

Fig 3. This illustrates the organization of the TcR α chain locus. (A) The constant region and the region of the J segments for 35 kb 5′ of the constant region. The region hybridizing to the constant region probe is depicted by ⊡ and regions hybridizing to J region probes are represented by ▨. This region was isolated by screening a human genomic DNA library in Charon 4A. λ clone 8 was isolated using a constant region probe. Probe A was used to isolate λ clone 21 and probe B was used to λ clone 22. C, D, E, and F are single copy probes isolated from λ clone 22. The restriction enzymes on the map are Bam HI (B), Eco RI (E), Hind III (H), and Xba (X). (B) The overall organization of the TcR α chain gene.

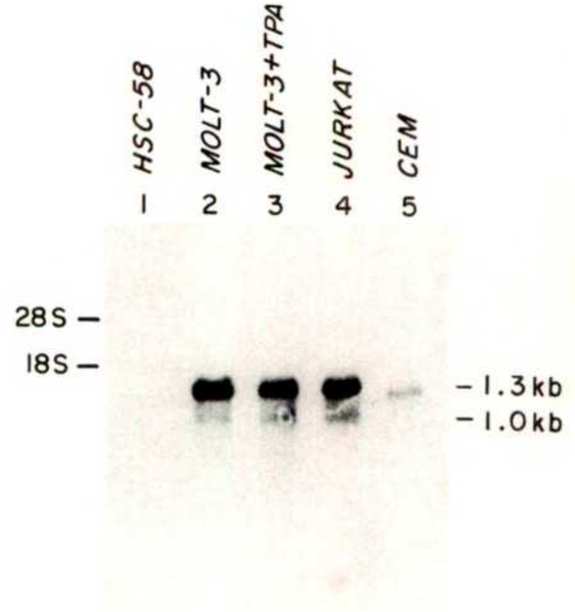

Fig 4. A Northern blot of mRNA extracted from five leukemic cell lines. HSC-58 is a B cell line and Molt 3+TPA Jurkat and CEM are all T cell lines. Poly A+ mRNA was separated by gel electrophoresis transferred to a nitrocellulose membrane and probed with a DNA fragment to the constant region of TcR β; both a 1.3 and 1.0 kb transcript can be seen.

cytoplasm to the nucleus. The functional unit of the T cell antigen receptor involved in this process has been found to consist of both the α and β chain genes of the TcR and at least three molecules of the T cell surface protein complex T3 or Leu4.[48-50] The expression of the TcR on the surface of T lymphocytes depends on several factors. First, there must be a complete and functional rearrangement, that is, a rearrangement that results in a V(D)J joining that encodes for a messenger RNA capable of producing a mature protein.[49,50] Second, the proteins for the T3/Leu4 must also be produced. The close association of these T3/Leu4 molecules and the α and β chain heterodimer has been demonstrated by coprecipitation of these molecules as well as by gene transfer experiments.[50] These later experiments not only demonstrate the comodulation of these molecules but also directly support the hypothesis that the α and β chain heterodimer is indeed the functional T cell antigen receptor (unpublished observation).[50]

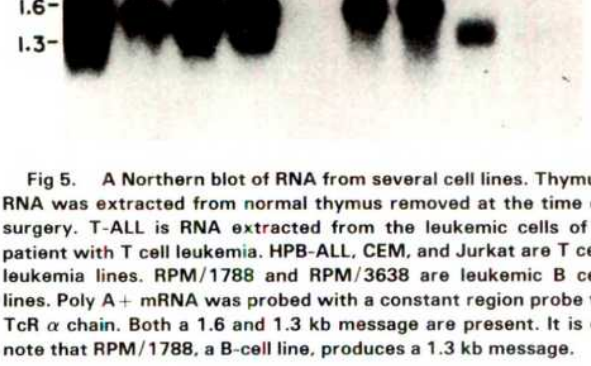

Fig 5. A Northern blot of RNA from several cell lines. Thymus RNA was extracted from normal thymus removed at the time of surgery. T-ALL is RNA extracted from the leukemic cells of a patient with T cell leukemia. HPB-ALL, CEM, and Jurkat are T cell leukemia lines. RPM/1788 and RPM/3638 are leukemic B cell lines. Poly A+ mRNA was probed with a constant region probe to TcR α chain. Both a 1.6 and 1.3 kb message are present. It is of note that RPM/1788, a B-cell line, produces a 1.3 kb message.

The Rearrangement of T Cell Receptor Genes in Hematologic Malignancies and Proliferative Diseases of the Lymph Node

Rearrangement of immunoglobulin genes in B cell malignancies has proven to be a useful marker for the B cell lineage.[51-53] In general the same can be said for rearrangement of TcR genes in T cell malignancies. Since the initial

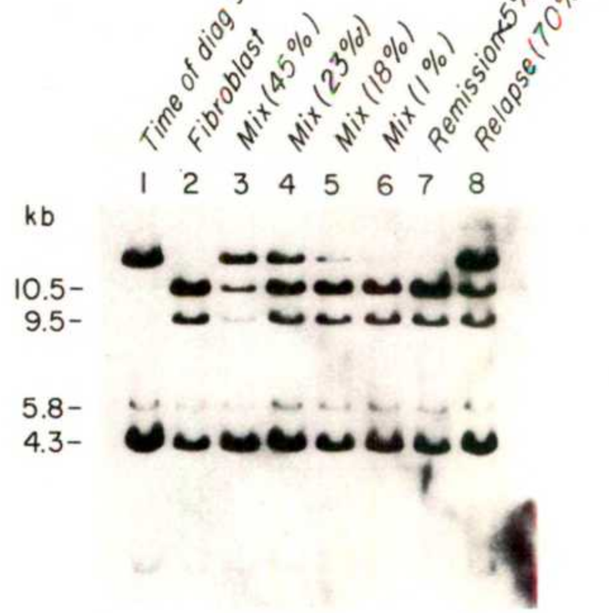

Fig 6. A Southern blot illustrating the sensitivity of the technique for detecting a clonal population. Lane 1 represents the sample drawn at the time of diagnosis and contained >90% blasts. Lane 2 contains normal fibroblast DNA from the same patient. Lanes 3, 4, 5, and 6 represent mixtures of the diagnosis DNA and fibroblast DNA; the percentage is the percentage of leukemic DNA in the sample. Lane 7 is a remission sample in which <5% blasts could be seen. Lane 8 is a relapse sample in which 70% of the cells were blast cells. The DNA was cut with Eco RI separated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with a constant region fragment of TcR β. The band characteristic of the leukemic cells can be seen in this sample consisting of 1% leukemic cells and 99% fibroblasts. The remission sample that contains <5% blasts does not contain a band characteristic of leukemic cells; thus, the number of leukemic cells in this sample is <1%.

Table 1. Use of Evaluation of TcR β Gene Structure in Lympho-Hematopoietic Malignancies

- Identification of a clonal population of cells
- In context of cell surface markers and structure of the immunoglobulin genes assign a lymphoid tumor to the T cell or B cell lineage
- Estimate extent of disease
- Identify early relapse of disease
- Identify progression of disease

cloning of the TcR genes, a large number of malignant and nonmalignant samples from patients with hematologic disorders have been examined.[26,43,44,54–64] The studies have confirmed that rearrangement of TcR β chain genes is a useful marker for the T cell lineage. Furthermore, analysis of the structure of these genes has been useful for establishing the clonality of the malignant cells in leukemia or lymphoma patients at the time of presentation or relapse. These studies are particularly useful in instances where there is a preponderance of morphologically normal T lymphocytes. In such cases, there is not a clear marker to indicate whether one is dealing with a clonal or polyclonal expansion of T cells. The finding of TcR rearrangement provides evidence that a clonal population exists. The sensitivity of detecting a clonal population has been tested. Using current hybridization technology, it is possible to detect a clonal population comprising 1% of the total population of cells. That is, if in a reconstruction experiment varying amounts of a sample that shows rearrangement is mixed with a sample that does not show rearrangement, one can detect the rearrangement if as little as 1% of the sample is DNA from the cells that contain a rearranged TcR β. To put this into perspective in terms of cell numbers, 10 μg of DNA is derived from 10^6 cells; therefore, if in this sample, 10^4 cells represent the clonal expansion of a population of T cells they will be detected by the presence of a rearranged band (Fig 6).[26]

The ability to detect clonal populations of T cells provides a means of monitoring the effect of chemotherapy, detecting early relapse, and identifying progression of disease not only in terms of percent replacement by tumor but also with regard to the emergence of new clonal populations of T cells (Table 1).

The results of studies carried out on a variety of T cell proliferative states are summarized below and in Table 2.

Table 2. Rearrangement of TcR β in Specimens from Patients With Leukemia or Lymphoma

	Number	Rearranged Tcr β	Rearranged Ig HC	Reference
T cell malignancies				
T-ALL	11	11	2	44
	12	12	1	26
	3	3	ND	54
	2	2	0	55
	3	3	ND	57
	2	2	ND	59
	5	5	0	61
	4	4	1	62
	17	17	5	77
ATL	2	2	ND	54
	1	1	ND	58
	4	4	0	61
	3	3	ND	59
T cell lymphoma	2	2	ND	54
	15	12	0	55
	4	4	ND	56
	5	5	ND	59
	3	3	0	62
	14	13	0	63
T cell prolymphocytic leukemia	1	1	ND	26
	2	2	0	55
	4	4	0	57
	5	5	0	62
	2	2	0	77
Sezary syndrome/ mycosis fungoides	2	2	ND	54
	1	1	0	55
	2	2	ND	57
	1	1	ND	58
Sezary syndrome/	20	19	ND	59
mycosis fungoides	14	14	ND	60
	5	5	1	61
	4	4	0	62
T-CLL	2	2	ND	54
	4	4	0	55
	5	5	ND	57
	1	1	ND	58
	12	12	0	62
T8 Lymphocytosis	3	2	ND	58
	1	0	ND	59
	1	1	0	61
B cell and Pre-B cell malignancies				
ALL	11	3	11	62
	6	0	6	26
	29	10	29	
Lymphoma	12	1	12	62
	7	2	7	63
B-CLL	10	2	10	61
	3	1	3	62
Myeloma	2	0	2	62
Other hematopoietic malignancies				
Lennerts	1	1	0	55
	5	5	0	63
HD	1	0	ND	59
	8	4	0	63
AIL	1	0		59
	11	9	4*	63
AML	6	0	0	26
	24	3	1*	64

*These cells also had rearrangement of TcR β.

Abbrev: T-ALL, T cell precursor acute lymphoblastic leukemia; ATL, Adult T cell leukemia; T-CLL, T-cell chronic lymphocytic leukemia; ALL, Common ALL non-B, non-T ALL, cALLa; B-CLL, B cell, chronic monocytic leukemia; HD, Hodgkin's disease; AIL, Angioimmunoblastic lymphadenopathy; AML, Acute myeloblastic leukemia; ND, not done.

T CELL MALIGNANCIES

T Cell Acute Lymphoblastic Leukemia (T-ALL)

The structure of the β chain of the TcR has been evaluated in over 30 cases of T-ALL.[26,43,44,55,57,61] In all cases rearrangement of one or both alleles of TcR was detected. Cell surface marker studies carried out on the same group of patients were heterogeneous in that no single marker was present in all cases. For example, cells with rearrangement of TcR did not always express the T3 cell surface antigen. In addition to rearrangement of TcR, some cases also had rearrangement of the Ig heavy chain but not Ig light chain genes. Rearrangement of TcR β was also present in adult T cell leukemia (ATL).

Chronic T Cell Malignancies

T cell malignancies such as CLL, mycosis fungoides, and Sezary syndrome have been evaluated.[26,43,55–61] In all of the cases studied there was rearrangement of the β chain of the TcR. In the case of mycosis fungoides the authors demonstrated that draining lymph nodes contained tumor cells.

T8 or T4 Lymphocytosis

T8 lymphocytosis or cytotoxic-suppressor lymphocytosis and neutropenia is a rare disorder with a benign course.[59,60] The structure of TcR β has been evaluated in five patients with this disorder. Three of the five patients showed the presence of a clonal population of T cells in their peripheral blood. Based on this, one of the investigators equated T8 lymphocytosis to the benign monoclonal gammopathies.[53] Certainly the chronic nature of this ailment indicates that the finding of a clonal population of cells does not necessarily indicate the presence of a malignant disease. The finding that not all patients with T8 lymphocytosis have a clonal population may be due to a technical artefact (see below), may indicate that the syndrome is heterogeneous, or may be due to the fact that the disease starts off being polyclonal but over time a single clone becomes dominant. Sequential studies are required to resolve this. In one patient that we have studied with T4 lymphocytosis and neutropenia, we found a predominant clonal population (unpublished result).

T Cell Lymphomas

Most if not all of the non-Hodgkin's lymphomas with T cell markers show rearrangement of the TcR β chain.[43,54–63]

B-CELL AND PRE-B-CELL MALIGNANCIES

Non-B, Non-T ALL

The structure of both the immunoglobulin genes and TcR β chain genes have been studied in non-B, non-T ALL.[44] In all of the cases rearrangement of the Ig heavy chain was found. This was taken as evidence that tumors were of the B cell lineage. However in a study of 29 such patients rearrangement of TcR β was found in 10 patients. The pattern of TcR rearrangement did not differ significantly from that in T cell malignancies.

Chronic B Cell Malignancies

Approximately 10% of patients with B-CLL have rearrangement of the TcR β gene.[61]

Non-Hodgkin's Lymphoma—B Cell Type

As in other B cell type malignancies, rearrangement of the TcR occurs in approximately 10% of the cases.[62,63]

OTHER HEMATOPOIETIC MALIGNANCIES

Acute Myeloblastic Leukemia

In a survey of 24 patients with AML, rearrangement of TcR β was found in 3 patients.[64] All of these patients expressed myeloid cell surface markers. In addition, two of the patients were positive for TdT and one of the patients was positive for the T cell surface marker T6.

Other Types of Lymphomas

There is a group of lymphomas consisting of Lennert's lymphoma, Hodgkin's disease, and angioimmunoblastic lymphadenopathy in which the presence of a clonal population of cells has not previously been clearly demonstrated.[55,63] We have investigated representative samples of each of these diagnoses.

Lennert's lymphoma. In 5 out of 5 cases of Lennert's lymphoma, rearrangement of the TcR β gene was observed. There was no rearrangement of the immunoglobulin genes.

Hodgkin's disease. Eleven cases of Hodgkin's disease were analysed. A faint but noticeable rearranged band was observed in four cases. One of the cases studied had approximately 50% Reed Sternberg cells; there was no rearrangement in this case. No rearrangement of immunoglobulin genes was observed. The fact that the rearranged bands were quite faint indicates that only a small proportion of the cells in the lymph node belong to the clone. It is not known at the present time whether this clonal population of cells represents the malignant cell or whether they represent a clonal proliferation in response to some antigen. That the cells carrying the rearrangement may be part of a malignant clone is strengthened by the finding that a cell line derived from a patient with Hodgkin's disease contains a rearranged TcR β.

Angioimmunoblastic lymphadenopathy (AIL). Eleven cases of AIL were examined; rearrangement of TcR β was found in 9 cases. In addition, 4 of the 9 cases showed rearrangement of the immunoglobulin heavy chain genes. One of these samples also showed rearrangement of the kappa light chain gene.

The above results indicate that in Lennert's lymphoma, Hodgkin's disease, and AIL there are clonal populations of cells that bear a rearranged TcR β gene. In the case of Lennert's lymphoma and AIL where these cells make up a major proportion of the lymph node, the finding of TcR β rearrangement may be taken as evidence that these are diseases of T cell origin. The finding of a minor population of cells with TcR β rearrangement in Hodgkin's disease requires further investigation.

The finding of rearrangement of TcR β in non–T cell malignancies raises the question of specificity of this finding in terms of defining the T cell lineage. With regard to using rearrangement of the TcR β to determine the lineage of a cell one must exercise caution and take into account other information such as the structure of the immunoglobulin genes and the expression of cell surface markers.

Involvement of TcR in the Development of Malignancy

Recurrent translocations have been observed in a number of tumors.[65] For example, in chronic myelogenous leukemia almost all patients have a characteristic translocation involving chromosomes 9 and 22,[66] and in B cell malignancies translocation involving the region 14q32 and some other chromosome are frequently seen, eg, chromosome 8.[67,68] The region 14q32 is the region of the heavy chain immunoglobulin genes while the region on chromosome 8 is the *myc* oncogene. In T cell malignancies recurrent translocations are also seen.[38,69–76] Translocations involving the region of the α chain gene 14q11-1.3 have been recognized; three such translocations are a reciprocal translocation involving 8q24 and 14q12,[76] an inversion involving 14q11 and 14q32,[71–73] and a reciprocal translocation involving 11p13 and 14q12.[38,73] In all three cases the α chain gene in the region of 14q11 to 14q13 has been disrupted. In the 8:14 translocation it has been shown that the translocation involves the V-region of the α chain and the region immediately 3′ of the c-*myc* proto-oncogene on chromosome 8.[76] This is the same region of the c-myc gene that is involved in the variant translocations observed in Burkitt's lymphoma.[68] In the chromosome 14 inversion involving band 14q12 and 14q32, the variable region of the Ig heavy chain locus becomes associated with the J region of the α chain locus.[77,78] In this case an oncogene is not involved in the translocation. It is interesting to note that this inversion is occasionally seen in normal T cells and in T cells from patients with ataxia telangiectasia.[79] The nature of the gene that resides in 11p13 and is involved in the translocation is not known. One possibility is that this might be a new oncogene whose expression or structure is altered as a result of the translocation. Such an alteration may play a role in the malignant transformation of the cell.

Rearrangement of TcR as a Marker of T Cell Ontogeny

In the B cell lineage it has been demonstrated that rearrangement of the immunoglobulin genes occurs in an ordered fashion.[51,80] In the T cell system it also appears that rearrangement and gene expression occur in an ordered fashion. The first gene to undergo rearrangement is the T cell gamma chain gene.[81–83] This is followed by rearrangement and expression of the TcR β chain. As mentioned previously, the earliest event appears to be DJ joining and the production of a 1.0kb mRNA. This is followed by VD joining and the production of a 1.3 kb mRNA. It has not been possible to demonstrate rearrangement of the α chain gene; however, expression of a mature, full length α chain mRNA occurs after the expression of β chain mRNA.[83]

Technical Considerations

The use of probes for the TcR β gene has proven useful in establishing clonality and lineage of T cell malignancies. The most information can be obtained by using a number of probes and restriction enzymes. The enzyme BAM HI in combination with a probe to the constant region is useful for detecting rearrangements involving either the C-$_{\beta1}$ or the C-$_{\beta2}$ regions. The normal germline band detected by this combination of probe and restriction enzyme is approximately 23 kb in length. This creates some difficulty as the resolution of such large pieces of DNA using conventional methods is poor. To overcome this problem we routinely run low percentage agarose gels of 0.7% to 0.8% at 2 volts per cm for 48 hours. This allows us to detect small differences in size in the 20 kb region. Advances in electrophoresis technology may overcome this problem.

The enzyme Eco R1 in combination with a constant region probe gives an 11 and 4 kb band. From the restriction map of TcR β the 11 kb band contains the C-$_{\beta1}$ region while the 4 kb band contains the C-$_{\beta2}$ region. DNA cleaved with Eco RI and probed with a constant region probe will only detect rearrangements of C-$_{\beta1}$. In analyzing such a study it is important to compare the intensity of the 11 kb and the 4 kb bands. In non-T cells these bands are of equal intensity. A decrease in the intensity of the 11 kb band can come about due to the presence of a polyclonal T cell population which has randomly rearranged or deleted C-$_{\beta1}$. In such a case, because the number of cells carrying the same rearrangement are below the level of detection (1% of the sample), one does not see new distinct bands but rather a decrease in the intensity of the 11 kb band. A second reason for a decreased intensity of the 11 kb band is that there is a clonal population in which C-$_{\beta1}$ has been deleted from one chromosome and is not rearranged on the other chromosome. This can be determined by evaluating the structure of the C-$_{\beta2}$ by probing DNA, cut with BAM HI or Hind III, with a constant region probe. Germline DNA cut with Hind III and hybridized to a constant region probe gives bands of 8.0, 6.5, and 3.5 kb. The 3.5 kb band contains the C-$_{\beta1}$ region while the 8.0 band contains the 5′ part of C-$_{\beta2}$. The 6.5 kb band contains the 3′ region of C$_{\beta2}$. As there is a Hind III site between the J of C-$_{\beta1}$ and its constant region, rearrangement of C-$_{\beta1}$is not detected; however, rearrangements into the J of C-$_{\beta2}$ are detected. An alternate strategy is to probe DNA cut with Eco RI with a probe to the J region of C-$_{\beta2}$. This results in a germline band of 4 kb; rearrangement into the Js of C-$_{\beta2}$ will result in an altered length of this fragment.[64]

At the present time the size of the J region of TcR α makes it very difficult to study rearrangement of this gene. Through the accumulation of more probes to the J region it should be possible to overcome this problem.

Summary

In this review the genomic structure and the RNA transcripts of the α and β chain of the T cell antigen receptor have been discussed. Studies of the structure of TcR β in hematologic malignancies have revealed rearrangement in almost all of the T cell malignancies and a small proportion of non–T cell malignancies. In addition, clonal involvement of T cells in diseases such as Hodgkin's disease, angioimmunoblastic lymphadenopathy, and chronic T cell lymphocytosis have been observed. The study of the structure of the TcR

β gene is thus a useful tool for identifying clonal expansions of cells and in conjunction with studies of the immunoglobulin gene structure, and cell surface markers a useful tool for identifying cell lineage. At the present time the evaluation of the structure of the α chain genes has not been as fruitful. However, chromosome translocations involving the TcR α chain genes have been recognized and, in one case, this rearrangement has been in association with a known oncogene. With the isolation of more probes to the α chain region it should be possible to test its utility in identifying clonal populations and cell lineage. The recent isolation of the gamma gene of the T cell will also permit such studies. Preliminary results of studies carried out with a probe to the γ chain gene of the T cell have paralleled results obtained with the TcR β probe (unpublished observation).

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Defective Platelet Adhesion on Vessel Subendothelium in Uremic Patients

By Ricardo Castillo, Teresa Lozano, Ginés Escolar, Luis Revert, José López, and Antonio Ordinas

Bleeding time, platelet retention on glass beads, and ristocetin-induced platelet agglutination (RIPA) in platelet-rich plasma were simultaneously determined for 20 patients with chronic renal failure and previous hemorrhagic history. In seven patients chosen at random out of a group of 16 in whom the three tests were abnormal, RIPA of uremic-isolated platelets in presence of normal platelet-poor plasma (PPP) and of normal platelets in presence of patient PPP were performed. In all cases, the first assay showed diminished agglutination, suggesting a platelet defect; however, uremic PPP did not inhibit the agglutination of normal platelets. In the same patients, the interaction of platelets with subendothelium was evaluated using Baumgartner's perfusion method. The subendothelial surface covered by platelets was significantly decreased in experiments with uremic whole blood when compared to normal controls. The interaction of platelets with subendothelium was also decreased when perfusions were carried out with platelet-plasma mixtures containing either normal washed platelets and uremic PPP or uremic washed platelets and normal PPP. These results show an impaired platelet adhesion caused both by a platelet and a plasmatic abnormality. Since uremic PPP decreased the adhesion of normal platelets to subendothelium but did not inhibit RIPA, it seems probable that the plasmatic defect could result in a defective binding between vWF and subendothelium. The influence of the red cell count on the platelet adhesion to subendothelium was reconfirmed by comparing perfusions of reconstituted blood with hematocrit values of 20% to 23% and 40% to 45%. In summary, a defective platelet adhesion to subendothelium has been postulated in uremic patients, caused by platelet and plasmatic alterations that are influenced by a low hematocrit.

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THE alteration of platelet function in uremic patients is common and often associated with a hemorrhagic tendency that is evidenced by a prolonged bleeding time.[1-8] In such cases an increase in the formation of prostacyclin and a decrease in thromboxane production have been found, indicating an unbalanced prostaglandin metabolism that may be considered as one of the causes of the increased hemorrhagic risk.[9-14] A defective ristocetin-induced platelet agglutination (RIPA) in platelet-rich plasma (PRP) has been also found in uremia.[15] Furthermore, the infusion of cryoprecipitate[4] and 1-deamino-8-D-arginine-vasopressin (DDAVP),[3] a synthetic derivative of antidiuretic hormone, has been shown to shorten the bleeding time in uremic patients.

In order to determine if a defect of platelet–von Willebrand factor (vWF) interaction played a role in the hemostatic abnormalities in uremic patients, RIPA of normal platelets in presence of uremic plasma, or uremic platelets in normal plasma were studied. Since RIPA does not measure the process of platelet adhesion to vascular subendothelium,[16,17] we studied adhesion to denuded vessel wall of platelets in uremic whole blood and mixtures of normal and uremic platelets in the presence of uremic and normal PPP, respectively.[18,19]

PATIENTS AND METHODS

Twenty patients (14 females and 6 males aged 27 to 63 years) with chronic renal failure and a previous history of bleeding had determinations of their bleeding time, platelet retention on glass beads, and RIPA in PRP performed. In addition, factor VIII activity (FVIII:C), von Willebrand factor antigen (vWF:AG), and ristocetin cofactor (FVIIIR:RCo) were measured in plasma and platelet malondialdehyde (MDA) production was determined after collagen stimulation.

In seven patients in whom bleeding time, platelet retention on glass beads, and RIPA were all abnormal, RIPA was also performed using patient platelets with normal plasma and normal platelets with patient plasma. Platelet membrane glycoprotein profiles and platelet sialic acid levels were determined and the interaction of platelets with the vascular subendothelium in the Baumgartner continuous perfusion system was evaluated, using whole blood and reconstituted blood with uremic-washed platelets or uremic PPP. At the time of the RIPA and the perfusion experiments, bleeding time and platelet MDA production were repeated.

Seven other patients were chosen at random from individuals undergoing regular hemodialysis who had no bleeding history. RIPA assays and perfusion studies were performed as described above.

Methods. Platelets were counted by phase-contrast microscopy. Bleeding time was measured using the "simplate"sterile disposable device (General Diagnostics, Morris Plains, NJ).[20]The normal range was 3 to 9 minutes. Platelet retention on glass beads was measured in native blood by a modification of Hellem's method[21] using standardized glass bead-filled columns at a constant rate of 1 mL blood/12 sec (Adeplot "S," Mascia Brunelli, Milano). The normal range was 60% to 90% retention.

Blood samples were obtained by venipuncture into citrated-phosphate-dextrose (CPD)[22] (resulting in a final plasma concentration of about 19 mmol/L citrate). PRP was prepared by centrifugation (150 g for 15 min) at room temperature. PPP was obtained after centrifugation (2,800 g for 20 min) at 4 °C and stored at −80 °C until tested.

RIPA was measured at 37 °C according to the technique of Born[23] using an aggregometer (Chrono Log, Haverstown, Pa). The initial velocity of agglutination was calculated by drawing a tangent to the steepest part of the agglutination curve on a potentiometric chart recorder, and expressed as the increase in transmission (measured in centimeters) after 30 seconds.[24,25] The final concentration of ristocetin (H. Lundbeck & Co, Denmark) was 1.2 mg/mL. In the RIPA studies with PRP, the platelet counts were higher than $1.2 \times 10^5/\mu L$ in all instances and each test was compared to normal PRP with approximately the same platelet count. In the RIPA assays of patient platelets with normal plasma and normal platelets with patient plasma, the platelet count was between 1.9 and $2.5 \times 10^5/\mu L$

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in all instances, and each test was compared with approximately the same concentration of normal platelets in normal plasma. The same normal PPP and platelets were used for both the control and the platelet-plasma mixtures. Both normal and patient platelets were isolated by gel filtration of PRP through a Sepharose 2B column (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Gel-filtered platelets were washed with Krebs-Ringer buffer[26] and tested to be sure that they would not aggregate with Ristocetin alone. The mixture of platelets and PPP was allowed to incubate for 90 minutes at room temperature before the addition of Ristocetin.

FVIII:C was assayed by the one-stage kaolin-activated partial thromboplastin time method.[27] vWF:AG was measured by quantitative immunoelectrophoresis according to Laurell[28] using rabbit antibody at 0.3% (Behring). FVIIIR:RCo was measured using freshly isolated platelets.[29] Platelet malondialdehyde production stimulated by collagen (10 ug/mL) was determined by the thiobarbituric acid method.[30]

Platelet membrane glycoproteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli[31] as previously described[32] and total platelet sialic acid was determined by the method of Jourdian et al,[33] using N-acetyl neuraminic acid as a standard.

Perfusion conditions. Perfusion was carried out at 37 °C with a perfusion chamber as developed by Baumgartner et al[18,19] with an effective annual width of 1.2 mm and a rod length of 7.2 cm. The flow was established by pumping blood through a Hemodialysis Blood pump (Renal Systems, Minneapolis, Minn) and the flow rate was fixed at 140 mL/min (800 sec^{-1} shear rate).

Approximately 14 mm long segments of chymotrypsin-digested rabbit abdominal aorta were inserted on the rod. After 10 minutes of perfusion at 37 °C, the segments were rinsed with phosphate-buffered saline and fixed with gluteraldehyde-formaldehyde. The fixed segments were sliced from the supporting rod, dehydrated with ethanol, embedded in JB-4 embedding material, thin-sectioned for light microscopy, and stained with toluidine blue.

Platelet deposition on subendothelium was quantified by the percentage of surface coverage (% of subendothelium covered by platelets) according to Baumgartner,[19] using a manual optical picture analysis system to obtain the morphometric parameters (MOP-20 Kontron, Messegerate GMBH, München, FRG). The mean value of this parameter using whole blood from healthy donors was 81.31 ± 4.99 (mean ± SD).

Reconstitution of the blood components was carried out by mixing 15 mL PPP, 5 mL washed platelets, and 20 mL washed red cells, to give a hematocrit value between 40% and 45%. Reconstituted blood with a hematocrit of 20% to 23% was obtained by mixing 26 mL PPP, 5 mL washed platelets, and 9 mL washed red cells. In both cases, the final concentration of platelets was between 1.9 to 2.5 × $10^5/\mu L$. For each patient perfusions of the patient's platelets with normal PPP and normal platelets with patient's PPP, those last two with a hematocrit set to 20% to 23% were carried out. The normal controls were performed with hematocrit values of 20% to 23% and 40% to 45%. The same normal elements were used for every test. The perfusate mixtures were incubated for 5 minutes at 37 °C before perfusions were performed.

Red blood cells were washed three times with saline containing 2% glucose. Isolated gel-filtered platelets were washed with Krebs-Ringer buffer according to Sakariassen et al.[34]

Statistical analysis. Student *t* test for independent data was used to compare the results of Ristocetin-induced agglutination and those of surface coverage by platelets on subendothelium in the perfusion system. Wilcoxon's test for paired data was used to compare the differences between surface coverage by platelets at both low and normal hematocrits of reconstituted blood with that obtained with normal whole blood.

RESULTS

All patients were on regular hemodialysis (14 m^2/h per week in thrice-weekly sessions). The dialysis unit used was Travenol, MPM, constant flow with cuprofane membranes. Tests were performed two hours prior to each hemodialysis session. The patients had not been transfused with blood or blood components, nor had they taken drugs known to affect platelet behavior for at least 2 months before the study. The range of blood urea nitrogen and creatinine levels was 50 to 100 mg/dL and 8 to 12 mg/dL, respectively, at the time of the studies. In all cases, the level of ionized plasma calcium was within normal ranges. Platelet counts were higher than 1.2 × $10^5/\mu L$ in all instances. The values of coagulation screening tests (prothrombin time, activated partial thromboplastin time, thrombin time, and fibrinogen) were normal.

Simultaneous determination of bleeding time, platelet retention on glass beads, and ristocetin-induced platelet agglutination in PRP. The three determinations were simultaneously performed on 20 patients; in 16 of them, a prolongation of the bleeding time, a defective platelet retention on glass beads, and a decreased RIPA were observed. Defective platelet retention was observed in all cases. Reduced RIPA was found in 19 patients and the prolongation of bleeding time occurred in 17 cases (Table 1).

In the same patients, platelet MDA formation was defective: 2.27 ± 1.9 umol/L/10^9 platelets (Control : 6.7 ± 2.3; Mean ± SD). On the other hand, the average of the results from the 20 patients for FVIII:C and vWF expressed in U/dL ± SE were: FVIII:C : 159 ± 19.35 (normal range:

Table 1. Simultaneous Determination of Bleeding Time, Platelet Retention on Glass Beads, RIPA of PRP, and MDA Platelet Production in Uremic Patients With Bleeding History

Patients	Bleeding Time (min)	Platelet Retention (%)	RIPA in PRP (% velocity)	MDA (μmol/L 10^9 platelet)
L.A.	18	4	31	4.8
D.A.	25	32	42	3.6
S.A.	18	11	77	3.7
J.A.	19	15	27	7.1
J.B.	6	15	48	0.6
C.C.	24	5	13	1.7
C.D.	14	7	8	2.0
A.D.	30	19	11	0.4
C.G.	13	49	35	0.4
A.G.	30	13	6	0.5
T.G.	16	32	30	1.8
P.J.	10	13	30	1.8
A.M.	23	18	55	0.6
J.R.	27	29	8	2.2
M.R.	8	40	54	2
J.S.	6	6	47	4.8
R.S.	12	7	16	2.8
C.T.	22	4	17	0.1
F.P.	28	22	10	1.9
P.P.	25	20	53	4
	Normal range 3-9	Normal range 60-90	Normal values 67 ± 11 (Mean ± SD)	Normal values 6.7 ± 2.3 (Mean ± SD)

Table 2. RIPA of PRP and of Uremic and Normal Platelets in Presence of Uremic and Normal PPP, Bleeding Time, and Platelet MDA Production in Patients With Bleeding History

	Ristocetin-Induced Platelet Agglutination (% velocity)				
Patients	Uremic PRP	Uremic Platelets + Normal PPP	Normal Platelets + Uremic PPP	Bleeding Time* (min)	MDA Platelet Production† (μmol/L/10^9 platelet)
A.G.	8	10	81	16	2.4
C.D.	9	26	100	28	1.8
C.G.	37	28	100	18	0.5
C.T.	18	20	88	19	0.2
C.C.	12	25	100	22	1.9
A.D.	10	21	90	24	0.4
J.R.	6	20	75	30+	2.1
Mean ± SD	14.2 ± 10.7	21.4 ± 5.9	90.5 ± 10.0		
		$P < 0.001$			

*Normal range: 3-9 min.

†Normal values (Mean ± SD): 6.7 ± 2.3.

52–150); vWF:AG: 205.5 ± 19.35 (normal range: 54–150); FVIIIR:RCo : 119.2 ± 8.86 (normal range: 60–130).

Ristocetin-induced agglutination of uremic platelets in presence of normal PPP, and of normal platelets in presence of uremic PPP. Out of the group of 16 patients in whom the bleeding time, the platelet retention on glass beads, and the RIPA in PRP were all abnormal, seven patients chosen at random were more extensively studied. Their platelets and plasma were tested for RIPA by mixing with normal plasma and normal platelets, respectively. In all cases, the patient uremic platelets showed a defective agglutination in the presence of normal PPP. In contrast, the agglutination of normal platelets in presence of uremic PPP was normal (Table 2). The difference was highly significant ($P < 0.001$). The results obtained with plasmas from 7 uremic patients chosen at random without bleeding history are presented in Table 3.

In all the patients the bleeding time was prolonged and the platelet MDA production was defective, but the platelet membrane glycoprotein profile and the platelet sialic acid levels were normal (data not shown).

Perfusion system. The results of percentage of subendothelial surface covered by platelets using whole blood from uremic patients was 10.1% ± 3.9 (Mean ± SD). All patients had a hematocrit between 20% and 23% (Table 4).

Using reconstituted blood with uremic-washed platelets, normal washed red cells, and normal PPP, the degree of surface covered by platelets was 18.0% ± 5.4 (Mean ± SD). The use of reconstituted blood with normal washed platelets, normal red cells, and uremic PPP showed 20.6% ± 5.3 of platelet surface coverage (Table 4). In these reconstituted studies, the final hematocrit was set between 20% and 23%. The results of the same tests performed in 7 uremic patients with no bleeding history are shown in Table 3.

The use of reconstituted blood with all the normal elements at a hematocrit of 20% to 23% showed the percentage of surface coverage by adhering platelets to be 41.2% ± 6.3 (Mean ± SD). The difference between this level and that obtained using uremic platelets or plasma was highly significant ($P < 0.001$; Table 4).

Finally, the subendothelial surface of the vessel covered by adhering platelets after perfusion of reconstituted blood with all the normal elements and a hematocrit of 40% to 45% was 68.5% ± 9.9 (Mean ± SD). When using the same elements but with a hematocrit of 20% to 23%, the difference in percentage of coverage is highly significant ($P < 0.001$; Table 4).

Six out of the 7 patients who had perfusion studies done with a hematocrit adjusted to 40% to 45% and using uremic platelets or plasma, showed an increase of the percentage of

Table 3. RIPA and Platelet Adhesion on Subendothelium in Uremic Patients With No Bleeding History

	Ristocetin-Induced Platelet Agglutination (% velocity)			Platelet Adhesion on Subendothelium (% surface coverage)*		
Patients	Uremic PRP	Uremic Platelets + Normal PPP	Normal Platelets + Uremic PPP	Uremic Whole Blood	Uremic Platelets + Normal PPP	Normal Platelets + Uremic PPP
P.G.	38	39	90	30.07	33	37
J.P.	25	28	87	11.4	18	25
A.A.	20	29	80	14.5	17	20
R.R.	22	28	85	24.8	29	33
R.A.	15	20	90	18.2	27	30
J.M.	33	35	95	17.8	23	28
M.C.	29	34	85	17.2	24	28

*In the tests with reconstituted blood, the hematocrit was set between 20% to 23%.

Table 4. Platelet Adhesion on Subendothelium, Bleeding Time, and MDA Platelet Production in Uremic Patients With Bleeding History

	Platelet Adhesion on Subendothelium (% surface coverage)								
Patients	Uremic Whole Blood (Ht 20%–23%)	Uremic Platelets + Normal PPP (Ht 20%–23%)	Normal Platelets + Uremic PPP (Ht 20%–23%)	Uremic Platelets + Normal PPP (Ht 40%–45%)	Normal Platelets + Uremic PPP (Ht 40%–45%)	Normal Platelets + Normal PPP (Ht 20%–23%)	Normal Platelets + Normal PPP (Ht 40%–45%)	Bleeding Time* (min)	MDA Platelet Production† (μmol/L/10^9 platelet)
A.G.	5	17	24	18	27	50	65	30	0.5
C.D.	11	8	25	10	28	38	66	14	2.1
C.G.	17	20	19	24	22	31	50	14	0.3
C.T.	11	25	19	29	22	40	70	22	0
C.C.	7	15	25	17	28	42	82	24	2.0
A.D.	8	22	20	24	24	48	72	23	0.3
J.R.	12	19	11	ND‡	ND‡	40	75	28	2.3
						$P < 0.01$			
Mean ± SD	10.1 ± 3.9	18.0 ± 5.4	20.6 ± 5.3			41.2 ± 6.3	68.5 ± 9.9		
				$P < 0.001$					
					$P < 0.001$				

The same normal platelets, FPP, and red cells were used for each patient in the different tests of platelet adhesion.

*Normal range, 3–9 min.

†Normal values (Mean ± SD) = 6.7 ± 2.3 umol/L/10^9 platelets.

‡ND, not determined.

surface covered by platelets to be 5% and 20% of the levels obtained at a hematocrit of 20% to 23% (Table 4).

DISCUSSION

The defects in RIPA demonstrated with uremic PRP, together with an abnormally prolonged bleeding time and decreased platelet retention on glass beads, indirectly suggest that the hemostatic abnormality in uremic patients is related to a defect in platelet adhesion. This hypothesis of a defective platelet adhesion to exposed subendothelium was substantiated by the results obtained with the Baumgartner perfusion system (Table 4, Fig 1).

Factors reported to be involved in the platelet adhesion mechanism are plasma proteins (mainly vWF but also fibronectin),[35,36] specific receptors on the platelet membrane,[37] different structures of the subendothelium (mainly

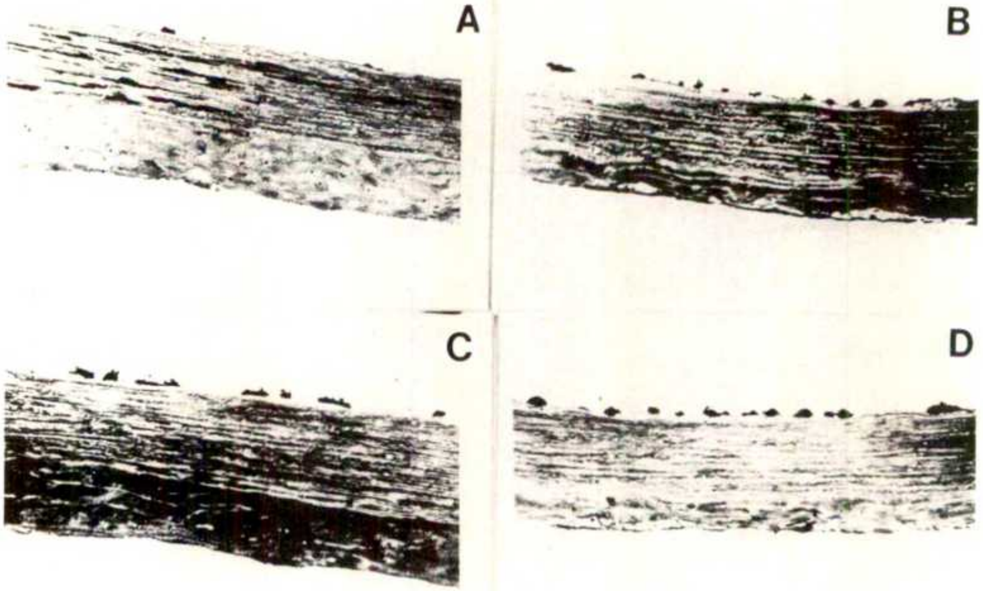

Fig 1. Light microscopical pictures of rabbit subendothelium showing surface coverage by adhering platelets after perfusion of uremic whole blood (A); reconstituted blood (RB) with normal red cells, normal PPP and uremic platelets (B); RB with normal red cells, normal platelets, and uremic PPP (C), and RB with all the normal elements (D). In all the tests the final hematocrit was set between 20% and 23%.

collagen),[38] and the concentration,[39,40] size, and rigidity[41,42] of the red cells. Changes in any of these factors could be partially or wholly responsible for the defective platelet adhesion observed in uremic patients.

The decrease in the percentage of subendothelial surface covered by platelets when reconstituted blood with uremic platelets or PPP was used in the perfusion system, suggests a defective platelet adhesion to the vessel subendothelium in uremic patients caused by both platelets and plasma.

Although the results of the abnormal platelet adhesion in uremics suggest that two components of the adhesion process (platelets and plasma) may coexist, both are probably due to the presence of toxic substances in plasma. These toxic substances could cause a qualitative alteration of glycoprotein I on the platelet surface or a blockage of the membrane von Willebrand receptor. In addition, they could induce an abnormality of the vWF molecule that is not detected by SDS-agarose electrophoresis,[3,43] or else prevent this protein from binding to glycoprotein I_b or to the exposed subendothelium of the vessel wall.

The defective agglutination observed in RIPA using uremic platelets and normal PPP suggests the existence of a membrane platelet defect that interferes with normal binding of vWF. However, since uremic plasma did not alter the RIPA of normal platelets, it seems probable that the plasma defect is more likely to be explained by the defective binding of vWF to components of the subendothelium.

The mechanisms that result in shortening of the bleeding time after infusions of cryoprecipitate[4] and DDAVP[3] in uremics are not known. One possible explanation could be that these products enhance the presence of the higher molecular weight multimers and/or the levels of vWF, thus overcoming the plasma effect.

No correlation between the existence of hemostatic alterations and the parameters of uremic dysfunction was observed. The levels of blood urea nitrogen (BUN) and creatinine were not related to the results of bleeding time, platelet retention on glass beads, perfusion studies, and platelet MDA production. No correlation between MDA production and other platelet tests was observed. A control group constituted by uremic patients undergoing hemodialysis but with no bleeding history was also studied (Table 3). Values of RIPA and platelet adhesion were consistently higher in this last group than in patients with hemorrhagic symptoms. These data would suggest that our observations have clinical relevance.

A significant negative correlation between the bleeding time and the hematocrit has been reported[44] and it is specially evident in uremic patients.[45,46] Moreover, and as demonstrated, not only the red cell count[39,40] but also their size and rigidity[41,42] play a role in the platelet adhesion to subendothelium.

The influence of the red cell concentration (hematocrit) on platelet adhesion to subendothelium of uremic platelets and plasma was confirmed in our studies with in vitro perfusion systems. The adjustment of the hematocrit to 40% to 45% using uremic platelets or plasma in 6 patients resulted in an increase in the surface covered by platelets of 5% and 20% of the levels obtained with the same components at a hematocrit of 20% to 23%. The effect of hematocrit also appears when reconstituted blood with all the normal elements at a hematocrit value of 40% to 45% is compared to blood also reconstituted with all the same normal elements but with a hematocrit of only 20% to 23% (Table 4). At low hematocrit the decrease of surface coverage was about 40% in normal controls. The low hematocrit also decreases the platelet adhesion to subendothelium in uremic patients, but it is platelet and plasma alterations that appear to play a major role. Perfusion studies with uremic platelets and plasma at hematocrit values adjusted to 40% to 45%, resulted in a partial but not a total correction of the number of platelets on the exposed subendothelial surface. On the other hand, the platelet adhesion using normal elements with a hematocrit of 20% to 23% was significantly higher than when uremic platelets or plasma with the same hematocrit were used.

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A Neutrophil Membrane Marker Reveals Two Groups of Chronic Myelogenous Leukemia and Its Absence May Be a Marker of Disease Progression

By John I. Gallin, Robert J. Jacobson, Bruce E. Seligmann, Julia A. Metcalf, Jean H. McKay, Ronald A. Sacher, and Harry L. Malech

An IgG$_1$ monoclonal antibody, 31D8, that recognizes normal neutrophil (PMN) membranes, was used to study PMN from patients with chronic myelogenous leukemia (CML). Nineteen patients with Philadelphia chromosome positive CML were followed over a ten-month period and compared with 23 normals, six patients with leukemoid reactions, and eight patients with phagocytic cell defects. The percentage of PMN binding of 31D8 among normal subjects was variable about a normal distribution with an average of 95 ± 2% of cells binding 31D8. In contrast, there were two groups of CML patients: in 14 patients 88 ± 3% PMN bound 31D8 while in the remaining five patients only 6 ± 6% PMN bound 31D8. PMN 31D8 binding was normal in the control patient groups. Control antibodies 7C3 (binds to PMN precursors) and OKM1 (binds to the CR3 (iC3b) receptor) bound normally to CML neutrophils. Functionally, CML cells had normal chemotaxis to several stimuli and normal superoxide generation to phorbol myristate acetate. However, superoxide production in response to fmet-leu-phe was significantly less in 31D8 negative CML PMN than both 31D8 positive CML PMN and normal PMN which contained 85% 31D8 positive and 15% 31D8 negative PMN. Clinically, 2 of 14 CML patients with 31D8 positive PMN were in blast crisis (one extramedullary) at the time of study and the other 12 patients remained clinically stable in the chronic phase during the ten months of study. In contrast, one of five patients with 31D8 negative PMN was in blast crisis at the time of study and all four of the remaining patients progressed to either the accelerated phase or blast crisis. Three of these patients died of their disease eight to ten months after their initial study. Thus, failure of CML cells to bind 31D8 may be useful for predicting which patients are likely to progress to the accelerated phase or blast crisis.

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HUMAN peripheral blood neutrophils have been shown to be heterogeneous by several parameters.[1] We have previously described an IgG$_1$monoclonal antibody that binds heterogeneously to human blood neutrophils.[2,3] The antibody, 31D8, bound strongly to most, but not all, neutrophils with considerable variability among people. Neutrophil expression of 31D8 antigen appeared early in myeloid maturation, about the myelocyte stage. Additional studies indicated the 31D8 positive neutrophils performed better than 31D8 negative neutrophils for adherence to glass, and *N*-formylmethionylleucylphenylalanine (fmet-leu-phe)-stimulated chemotaxis and oxidative metabolism. In addition, the 31D8 positive cells exhibited a membrane potential depolarization when stimulated with FMLP while the 31D8 negative cells did not.

It is not known why there are two populations of neutrophils nor the origin of the heterogeneity in normal subjects.[1] As part of our studies of the potential clinical importance of 31D8 expression, we initiated studies in patients with chronic myelogenous leukemia (CML). In this paper we present data that indicate that the majority of CML patients have normal neutrophil expression of 31D8 but in a minority (5 of 19 patients) nearly 100% of the neutrophils were 31D8 negative. Furthermore, the data indicate that failure of neutrophil expression of 31D8 antigen may be an early marker of progression of CML to the accelerated phase or blast crisis.

MATERIALS AND METHODS

Subjects. Nineteen patients with Philadelphia chromosome positive CML were studied over a 10-month period. Ten patients were females, three in blast crisis at the time of study, one extramedullary without blast cells detected in the blood or marrow. Nine patients were males. In addition, 23 normal subjects were studied in parallel, 12 females and 11 males. Control patients included 6 with leukemoid reactions with white blood counts ranging from 15,000 to 85,000 cells/mm^3. Eight patients with phagocytic cell defects were also studied including four patients with chronic granulomatous disease, 1 patient with neutrophil myeloperoxidase deficiency, one patient with neutrophil-specific granule deficiency, and one patient with absent macrophage apolipoprotein E production.[4-7]

Cell preparation and labeling with antibodies. Blood neutrophils were prepared by Hypaque-Ficoll and dextran sedimentation as described previously.[3] Neutrophils were incubated with fluoresceinated mouse antihuman monoclonal antibody 31D8 for 30 minutes at 4 °C and then washed ×3 with Hanks' balanced salts solution as previously described. The cells were then fixed with 2% paraformaldehyde and saved for study in the fluorescent activated cell sorter. As recently described, 31D8 negative cells had slightly more fluorescence (less than 10%) than unstained cells whereas 31D8 positive cells exhibited at least 5 to 10 times greater fluorescence than unstained cells.[3] In patients in blast crisis, identification of neutrophil precursors was done using parallel studies with a neutrophil-specific antibody we defined earlier called 7C3.[8] In other control studies the commercially available mouse antihuman neutrophil antibody OKM1 was utilized.[9] Wright's stain was performed on cytocentrifuge preparations of all cells prepared for analysis in the fluorescent-activated cell sorter and differential counts were performed.

Neutrophil function. Neutrophil chemotaxis to buffer, fmet-

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leu-phe (10^{-6} mol/L), endotoxin-activated serum (5% v/v), and sodium caseinate (5 mg/mL) was assayed by determining the average distance migrated by cells migrating into cellulose nitrate filters using a previously described technique.[6] Superoxide generation in response to phorbol myristate acetate (PMA, 20 ng/mL) and fmet-leu-phe (10^{-6}) was determined as the maximal rate of superoxide dismutase inhibitable cytochrome *c* reduction.[6]

Statistical analysis. Except where indicated standard errors were used as an estimate of variance and Student's *t* test was used to determine the significance of differences.

RESULTS

31D8 binding of CML cells. The percentage of 31D8 positive cells was determined in CML and normal subjects (Fig 1). Normal neutrophils can exhibit two populations of cells, the majority of which are 31D8 positive and the rest 31D8 negative (Fig 1A). Two patterns were observed in neutrophils from CML patients. In one group the majority of cells bound 31D8 normally (Fig 1B) while in the other the majority did not bind 31D8 (Fig 1C). Figure 2 shows the distribution of the percentage of 31D8 positive neutrophils among normals and patients. The two groups of CML patients consist of subjects having greater than 60% of their neutrophils binding 31D8 (mean 88 ± 3%) and those with less than 30% of their cells binding 31D8 (mean 6 ± 6%, $P < 0.001$ *v* neutrophils from normal subjects or patients with 31D8 positive cells). The width of the gap between the two apparent distributions of CML data was larger than would be expected by chance alone ($P < 0.02$ for a normal distribution). Although some CML patients had low percentages of neutrophils binding 31D8, the intensity of 31D8 binding to 31D8 positive and negative cells was normal (data not shown). Previously we showed that 31D8 binding normally appears about the myelocyte stage.[3] Differential counts on the cell preparations used for 31D8 binding studies indicated that the low percentage of 31D8 positive cells on some CML patients could not be accounted for by the overwhelming presence of immature neutrophils in these preparations. Four of five CML subjects with almost 100% 31D8 negative neutrophils had normal percentages of mature neutrophils, while the fifth patient, who was in blast crisis and had 0% 31D8 positive cells, had 17% peripheral blood neutrophil forms beyond the myelocyte state of maturation. 31D8 binding to neutrophils from patients with leukemoid reactions and neutrophil dysfunction was normal (93 ± 3% and 87 ± 5%, $P > 0.05$), and none of the patients with leukemoid reaction or with neutrophil dysfunction had a very low percentage of cells binding 31D8.

Binding of two other monoclonal antibodies, 7C3, an antibody that recognizes neutrophil lineages[8] and OKM1, an antibody that binds to the α chain of the CR3 (iC3b) receptor,[9] were also studied (Fig 3). Although there was some variability of 7C3 and OKM1 binding to CML neutrophils, unlike binding of 31D8, no CML patient neutrophils exhibited severe deficiency of 7C3 or OKM1 binding. Thus, the extreme heterogeneity of 31D8 binding to PMN from different CML subjects was specific for the 31D8 antibody with the three antibodies studied.

Neutrophil chemotaxis and superoxide generation. Eighteen studies of spontaneous nondirected locomotion to buffer and chemotaxis toward endotoxin-activated serum, fmet-leu-phe, or casein were performed on neutrophils from CML patients and concurrently run normals. With each stimulus tested the locomotive response to neutrophils from CML patients with greater than 60% 31D8 positive and less than 30% 31D8 negative neutrophils did not differ from normal cells (containing an average of 85% 31D8 positive and 15% 31D8 negative cells)[3] or from each other ($P > 0.05$). Superoxide generation was normal in neutrophils from all CML patients when PMA was the stimulus, but when fmet-leu-phe was used as the stimulus a small yet significant impairment of superoxide generation in neutro-

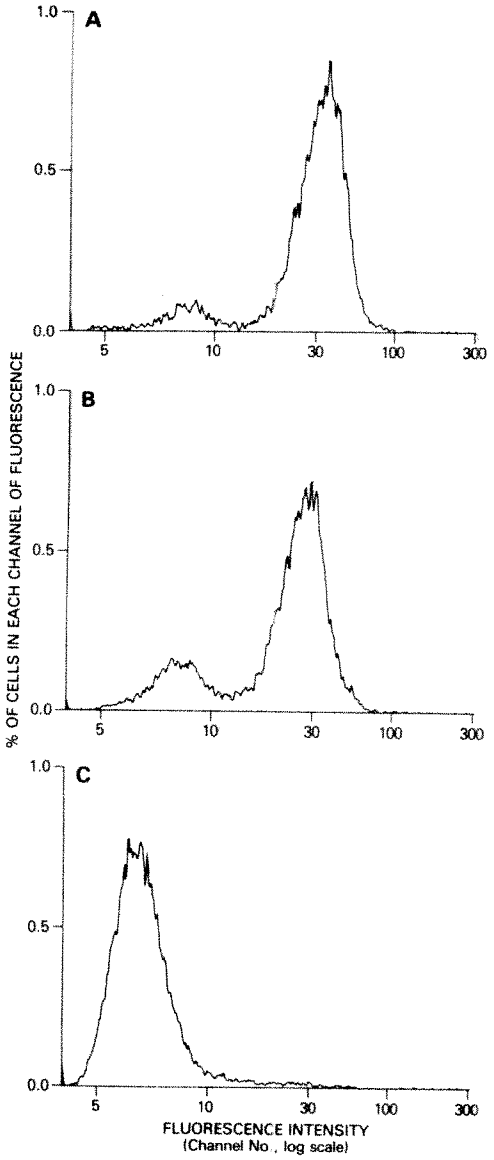

Fig 1. Histograms of 31D8 binding to neutrophils from a normal subject (A) and two patients with CML (B and C). Data obtained from the fluorescent activated cell sorter as described in the methods. All cells exhibited light scatter characteristic of neutrophils. Note 95% normal neutrophils in Panel A, 90% of CML cells in Panel B, and 0% of CML cells in Panel C were 31D8 positive (greater than 15 units of fluorescence). 10,000 cells counted for each histogram.

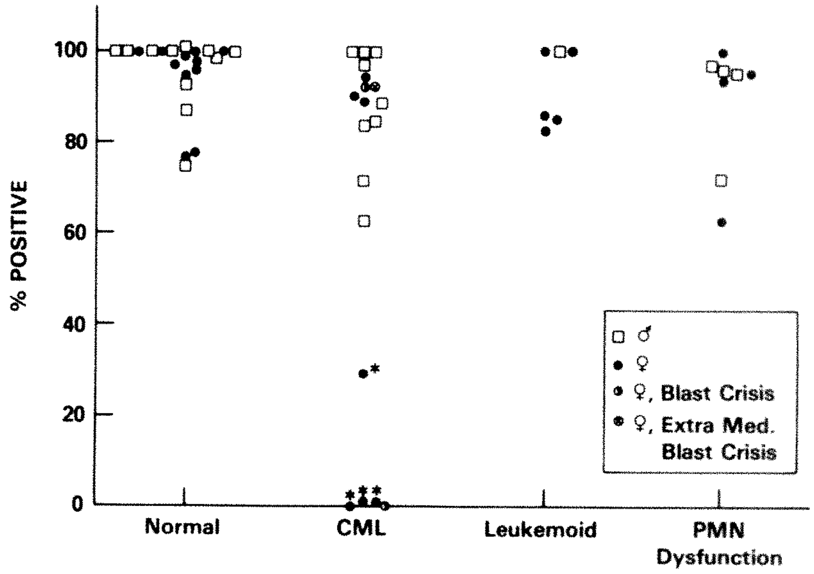

Fig 2. 31D8 binding to neutrophils from normal subjects and patients with CML, leukemoid reactions, and neutrophil dysfunction (see methods). Data are the mean percentage of cells binding the antibody as determined by flow cytometry, 10,000 cells counted. * Subsequently evolved to blast crisis or accelerated phase.

phils obtained from patients with 31D8 negative neutrophils (Fig 4) was seen. The magnitude of the impaired superoxide generation was similar to that noted in normal 31D8 negative neutrophils when compared with normal 31D8 positive neutrophils[3] but much less than that noted in patients with chronic granulomatous disease.[4]

Clinical characteristics of CML patients. Of 14 CML patients with 31D8 positive neutrophils, two were in blast crisis at the time of study, one being in extramedullary but not peripheral blood blast crisis. None of the other patients progressed to the accelerated phase or blast crisis during the 10 months of study. Of 5 CML patients with 31D8 negative neutrophils, one was in blast crisis at the time of study. However, of the remaining four patients with 31D8 negative neutrophils, three progressed to the accelerated phase and one progressed to blast crisis within 6 months of initial study. Three of the five patients with 31D8 negative neutrophils expired 8 to 10 months after initial study. A two-by-two contingency analysis of the clinical features of the CML patients indicates that these differences in the number of patients in blast crisis among the two patient groups were highly significant ($P = 0.002$, Fisher's Exact Test).

DISCUSSION

The data in this paper indicate that, based on binding of the monoclonal antibody 31D8 to blood neutrophils, CML patients can be divided into two groups: those with neutrophils expressing normal 31D8 antigen and those with neutrophils lacking 31D8 antigen. The 31D8 negative neutrophils seen in some CML patients expressed normal amounts of another myeloid antigen, 7C3, showing specificity of the defect. The differences in 31D8 antigen expression in the two patient groups cannot be attributed to therapeutic regimens.

An analysis of the clinical course of the disease in two groups of CML patients was of interest. The 14 patients with 31D8 positive neutrophils included two patients in blast crisis, one of whom was in extramedullary blast crisis. All the

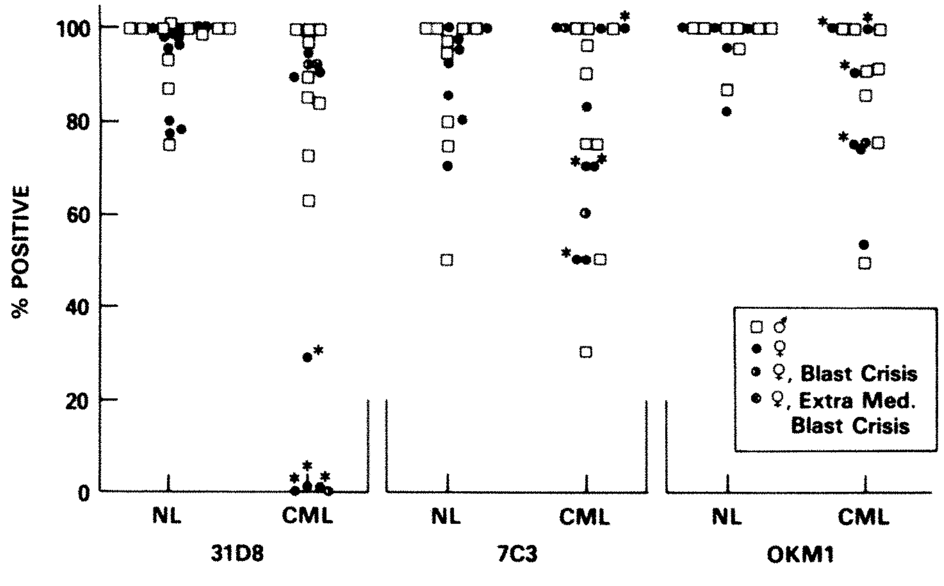

Fig 3. Binding of monoclonal antibodies to CML neutrophils. Data are the mean percentage of neutrophils binding the indicated antibody as determined by flow cytometry, 10,000 cells counted. * Subsequently evolved to blast crisis or accelerated phase.

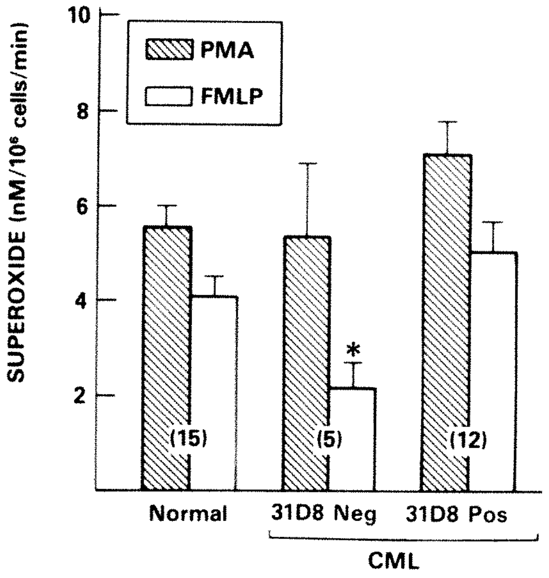

Fig 4. Maximal rates of superoxide production by normal and CML neutrophils stimulated with either PMA (20 ng/mL) or fmet-leu-phe (10^{-6} mol/L). Data are the mean ± SEM of the data obtained from the number of subjects shown in parentheses. 31D8 negative and positive CML neutrophils are as defined in Fig 1 and text. * $P < 0.01$ compared to FMLP CML 31D8 positive, $P < 0.05$ compared to FMLP normal.

patients in the chronic phase remained clinically stable during the 10 months of study and none had 31D8 negative cells. In contrast, of the five patients with 31D8 negative neutrophils, one was in blast crisis at the time of study, three progressed to the accelerated phase, and one progressed to blast crisis within 10 months of initial study. Thus, all five CML patients in the 31D8 negative category either were in, or progressed to, the accelerated phase or blast crisis.

It is of interest that the locomotive responsiveness to numerous stimuli and superoxide generation in response to PMA of CML 31D8 positive and negative neutrophils were normal. The normal chemotactic responsiveness of CML 31D8 negative neutrophils was different from normal 31D8 negative neutrophils which have impaired chemotaxis compared with normal 31D8 positive cells.[3] However, superoxide generation in response to fmet-leu-phe by cells from CML patients with 31D8 negative neutrophils was less than the response of cells from CML patients with 31D8 positive cells, similar to the results obtained between normal 31D8 positive and negative neutrophils.[2,3]

The current data are compatible with the concept that heterogeneity of PMN 31D8 antigen expression results from different stem cell pools, yet the data by no means establish this as correct. However, it is intriguing that failure to express 31D8 antigen on neutrophils from patients with CML may be an early indicator of disease progression, although it is clear that some patients can progress clinically yet still express 31D8 antigen on their neutrophils. Although the number of patients in this study are too small to reach definitive conclusions, the possibility failure of neutrophil expression of 31D8 antigen is of prognostic use in most patients with CML is of sufficient clinical importance to justify intense clinical evaluation of 31D8 expression in CML, particularly with the improving results with bone marrow transplantation.

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Studies on the Pathophysiology of Posttransfusion Purpura

By Thomas S. Kickler, Paul M. Ness, Jay H. Herman, and William R. Bell

Posttransfusion purpura typically occurs in PL^{A1} negative blood recipients who have been previously immunized to the PL^{A1} antigen. Following transfusion, severe thrombocytopenia develops with the formation of anti-PL^{A1}. Since the patients' platelets lack the PL^{A1} antigen, one would not expect this antibody to destroy autologous platelets. In this study we show that PL^{A1} antigen exists in stored blood and can absorb to PL^{A1} negative platelets making them PL^{A1} reactive. Incubating PL^{A1} (−) platelets with ultracentrifuged plasma from PL^{A1} (+) blood donors allowed anti-PL^{A1} to bind to PL^{A1} (−) platelets. Control plasma from PL^{A1} (−) blood donors did not lead to anti-PL^{A1} binding. Using an inhibition assay, we showed that stored blood contains PL^{A1} material that was not removed by ultracentrifugation. The material absorbing to PL^{A1} (−) platelets represented the PL^{A1} antigen, which was confirmed by Western blotting. After incubating plasma containing PL^{A1} antigen with PL^{A1} (−) platelets, reactivity at 95,000 D was observed. Native PL^{A1} (+) platelets showed a similar band. When PL^{A1} (−) platelets were incubated with plasma from a PL^{A1} (−) donor, this band was not present. These studies show that a soluble form of PL^{A1} antigen exists in stored blood that can absorb to PL^{A1} (−) platelets. Consequently, anti-PL^{A1} can bind to these platelets leading to thrombocytopenia. These observations may explain the autologous destruction of platelets in posttransfusion purpura.

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THE syndrome of posttransfusion purpura (PTP) was initially described by Shulman and coworkers.[1] This disorder typically occurs in patients whose platelets lack the platelet-specific antigen PL^{A1}, although other antigens have also been incriminated. Van Loghem and coworkers had originally characterized this platelet antigen system and named it Zw^{a}.[2] Thrombocytopenia occurs after transfusion with PL^{A1} positive blood in individuals previously sensitized by pregnancy or transfusion. With the development of thrombocytopenia, anti-PL^{A1} is found in the patient's blood. The perplexing question is why the patient's own PL^{A1} (−) platelets are destroyed. One would not expect the platelet alloantibody to destroy autologous platelets since they lack the PL^{A1}antigen.

Various mechanisms have been proposed to explain the induction of PTP. These include the production of autoantibodies[3] or immune complex-mediated platelet destruction as occurs in drug-induced immune thrombocytopenia.[4] A third explanation is that transfused antigenic material adheres to the PL^{A1} (−) platelets permitting the binding of antibody with resultant platelet destruction. This possibility is suggested by the reported ability of platelets to absorb blood group antigens and HLA antigens.[5-8]

This report presents experimental results supporting the hypothesis that PL^{A1} (−) platelets can become PL^{A1} reactive by absorbing transfused PL^{A1} reactive material.

MATERIALS AND METHODS

Antisera. Anti-PL^{A1} was obtained from a PL^{A1} (−) woman 2 months following recovery from PTP. Another example of anti-PL^{A1} was obtained from a mother of a child with alloimmune thrombocytopenia. The anti-PL^{A1} sera reacted at a titer of 1:256 with PL^{A1} (+) platelets.

Platelets. Group 0, PL^{A1} (−) platelets were prepared from EDTA anticoagulated blood.[9] The platelets were then washed four times with Tyrode's-EDTA, 3% BSA buffer. The platelet count was adjusted to 200,000/mm^{3}.

Radiolabeled antiglobulin test to measure anti-PLA1 binding. Anti-PL^{A1} binding to platelets was measured using a radiolabeled antiglobulin technique.[9,10] The anti-PL^{A1} binding is measured using affinity purified ^{125}I goat antihuman IgG labeled by the chloramine T method.[11]

Transfer of PLA1 reactivity to PLA1(−) platelets. To determine if PL^{A1} (−) platelets can absorb PL^{A1} antigenic material from stored blood, the following were done. Plasma was obtained from units of CPD-A1 anticoagulated PL^{A1} (+) whole blood that had been stored 5 to 7 days. The plasmas were centrifuged at 100,000 g for 30 minutes to remove any particulate material. Two mL of the plasma were incubated with 4 × 10^{8} platelets from PL^{A1} (−) individuals for 12 hours at 22 °C. Following the incubation, platelets were washed using Tyrodes-BSA buffer.

These platelets were then assayed for their ability to bind anti-PL^{A1} using the indirect radiolabeled antiglobulin test. For negative controls, PL^{A1} (−) platelets were incubated with plasma obtained from stored whole blood from a PL^{A1} (−) donor, or with buffer.

For an additional control, red cells were substituted for platelets to exclude the possibility that observed anti-PL^{A1} binding was related to trapping of platelet membrane debris or microparticles.

Inhibition assay to measure PLA1 antigenic material in stored blood. To determine the frequency with which stored blood contains a plasma form of PL^{A1} antigenic material, an inhibition assay to measure PL^{A1} antigen was devised. A total of 25 stored units were tested.

The test plasma was centrifuged at 100,000 g for 30 minutes. One mL of this plasma was incubated for 30 minutes at 37 °C with 1 mL of anti-PL^{A1} that had been diluted to a titer of 1:128. The mixture was then added to 2 × 10^{8} platelets to determine the amount of free anti-PL^{A1} remaining that could bind to the platelets as determined by the radiolabeled antiglobulin test. The inhibition of the anti-PL^{A1} binding after incubation with test plasma from stored PL^{A1} positive blood was compared to the inhibition when plasma from a PL^{A1} (−) plasma was used. The source of this plasma was from an untransfused PL^{A1} (−) donor. The lack of inhibitory activity of the PL^{A1} (−) plasma was also confirmed by comparison to the inhibition caused by buffer.

Western blotting. To characterize the material binding to PL^{A1} (−) platelets, we analyzed the converted platelets using Western blotting. After transferring PL^{A1} positivity to negative platelets, 2 × 10^{9} platelets were solubilized in 3% SDS preparation for SDS-polyacrylamide gel electrophoresis according to the method of McMillan.[12]

SDS-Polyacrylamide gel electrophoresis. The solubilized platelets were electrophoresed using the method of Laemelli.[13] Slab gels

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Table 1. Acquisition of Anti-PL^{A1} Binding by PL^{A1} Negative Platelets

Conditions						
Platelets		Plasma	Experiment 1	Experiment 2	Experiment 3	Experiment 4
PL^{A1}(−)	+	PL^{A1}(+)	9,688* ± 235	8,183 ± 311	6,710 ± 119	7,995 ± 260
PL^{A1}(−)	+	PL^{A1}(−)	3,567 ± 148	3,761 ± 91	3,891 ± 81	4,191 ± 119
PL^{A1}(−)	+	0	3,449 ± 58	3,982 ± 37	3,610 ± 44	4,221 ± 71
PL^{A1}(+)	+	0	11,565 ± 74	12,243 ± 52	12,049 ± 27	14,919 ± 91
Red Cells	+	PL^{A1}(+)	540 ± 69	300 ± 78	645 ± 30	417 ± 63

*The results of anti-PL^{A1} binding are given as mean counts per minute of radioactivity ± SEM measured by the radiolabeled antiglobulin test. Four different PL^{A1}(+) plasmas were used. The anti-PL^{A1} used was obtained from a patient who recovered from posttransfusion purpura.

of 1.5 mm thickness and 6% acrylamide were used with a 3% stacking gel. Molecular weight standards (Sigma, St. Louis, Mo) were run with each experiment.

Protein transfer to nitrocellulose paper. Proteins were electrophoretically transferred from gels to nitrocellulose paper using the method of Towbin.[14] Completeness of transfer was documented by fast green staining of the nitrocellulose.

Incubation of radiolabeled IgG with nitrocellulose immobilized proteins. Prior to incubation with anti-PL^{A1} serum, the immobilized proteins on the nitrocellulose strips were immersed for three hours in 3% BSA and 10 mmol/L Tris, .15 mol/L NaCl, .1% Tween 20. The anti-PL^{A1} serum was incubated one hour at 25 °C with the nitrocellulose paper. After washing the strips, with 3% BSA and .1% Tween 20 in mmol/L Tris, they were incubated with ^{125}I anti-IgG for one hour followed by additional washing to remove unbound radioactivity.

RESULTS

Conversion experiments. The ability of PL^{A1} (−) platelets to acquire the capacity to bind anti-PL^{A1} is shown in Table 1. When PL^{A1} (−) platelets were incubated with plasma from four different units of stored PL^{A1} (+) blood, anti-PL^{A1} binding was detected in all cases. The plasmas used as a source of PL^{A1} antigen were from 1-, 2-, 7-, and 10-day-old stored blood. Anti-PL^{A1} binding was 1.8 to 2.7 times greater than the negative controls. However, the amount of measured anti-PL^{A1} binding to the PL^{A1} (−) platelets after incubation did not equal the amount of anti-PL^{A1} binding to PL^{A1} (+) platelets. Since the anti-PL^{A1} did not react with PL^{A1} (−) platelets until incubated with plasma from PL^{A1} (+) plasma, PL^{A1} (−) platelets must acquire the ability to bind anti-PL^{A1} from some factor present in the plasma. Since all experiments used ABO compatible platelets and plasma and since PL^{A1} (−) plasma did not promote anti-PL^{A1} binding, the results show that PL^{A1} (−) platelets can absorb PL^{A1} antigenic material.

When red cells are substituted for platelets, no binding of anti-PL^{A1} to the red cells is observed. This suggests that the anti-PL^{A1} binding was unrelated to trapping of platelet membrane debris or microparticles. When anti-PL^{A1} from a patient with neonatal alloimmune thrombocytopenia was used instead of anti-PL^{A1} from a patient who recoved from posttransfusion purpura, similar results were obtained.

Inhibition assay results. To determine if most units of stored blood from PL^{A1} (+) donors contain PL^{A1} antigenic material in the plasma, we studied 25 randomly selected units of stored blood and analyzed ultracentrifuged plasma using an inhibition assay. The results of this analysis are shown in Table 2. Negative controls consisted of plasma from a PL^{A1} (−) donor who had never been immunized, and freshly collected plasma from PL^{A1} (+) donors. In Table 3, the results of a representative inhibition assay are shown.

All test plasmas obtained from stored units of blood promoted greater than 54% inhibition in anti-PL^{A1} binding to PL^{A1} (+) platelets. The amount of inhibition was similar for short-term stored units (one to three days) and units stored for longer time (seven to ten days). The specificity of the inhibition assay was shown by the lack of inhibition when plasma from a PL^{A1} (−) donor was used. These results show that all stored units contain material that can bind to PL^{A1} (−) platelets as described above.

Western blotting results. Converted platelets were also studied using Western blotting to identify PL^{A1} antigenic material. Plasma capable of promoting anti-PL^{A1} binding, as measured by the radiolabeled antiglobulin test, was incubated with PL^{A1} (−) platelets. A control consisted of using plasma from a PL^{A1} (−) donor. After incubating these plasmas with PL^{A1} (−) platelets, the platelets were solubilized and run in parallel with PL^{A1} (+) and PL^{A1} (−) platelets. The resultant autoradiograph is shown in Fig 1. One can see that when plasma from a PL^{A1} (+) donor is used, a band at 95,000 daltons is observed which is not detected when the PL^{A1} (−) platelets are incubated with plasma from a PL^{A1} (−) donor. This band corresponds to that observed with native PL^{A1} (+) platelets and is the reported molecular weight of PL^{A1}.[22] The nonspecific reactivity at 210,000, present in all lanes, is enhanced by incubation with plasma (lanes A and B), probably the result of absorbed plasma IgG. When the Western blot was developed using normal AB plasma in place of anti-PL^{A1} plasma, no bands in the region corresponding to the 95,000 Dalton band were observed. These results provide further support that PL^{A1} antigenic material is present in plasma.

DISCUSSION

Central to the mystery of posttransfusion purpura is why patients destroy their own platelets that lack the antigen to which they have been sensitized. Shulman et al[1] have pointed out the similarity to drug-induced purpura, where there is an

Table 2. Measurement of PL^{A1} Reactive Material in Plasma From Stored Blood Using an Inhibition Assay

Source of Plasma	Percent Inhibition
1- to 3-day-old PL^{A1}(+)	68.0 ± 7, n = 9
4- to 7-day-old PL^{A1}(+)	67.0 ± 9, n = 8
8- to 9-day-old PL^{A1}(+)	73.0 ± 6, n = 8
Freshly collected PL^{A1}(+)	13.0 ± 4.2, n = 5
PL^{A1}(−)	0

Table 3. Results of a Representative Inhibition Assay

	Counts Per Minute of Measured Anti-PL^{A1} Binding (experiment performed in triplicate)		
Plasma from PL^{A1}(+) Donor	6,045 (62%)	5,710 (68%)	4,581 (62%)
Plasma from PL^{A1}(−) Donor	15,885	17,703	17,321
Buffer	15,053	16,691	15,615

The inhibition assay used plasma centrifuged at 100,000 g for 30 minutes; this was incubated with anti-PL^{A1}, diluted 1:128, for 30 minutes. PL^{A1}(+) platelets were then added and the amount of anti-PL^{A1} binding was determined using the radiolabeled antiglobulin test. The percent inhibition is given in parenthesis.

abrupt onset of thrombocytopenia, followed by recovery despite persistence of the inciting antibody. These investigators proposed that PL^{A1} antigen-antibody complexes bind to platelets in a similar fashion to drug purpura as one possible mechanism for PTP.

Serologic studies during the acute phase of PTP have demonstrated the presence of increased levels of IgG on the surface of the patient's platelets.[10,15] Following elution of this IgG activity, Pegels showed anti-PL^{A1} specificity with strong reactivity against known PL^{A1} (+) platelets. They also observed weak reactivity with PL^{A1} (−) autologous platelets from patients in remission. They interpreted these results to indicate the presence of PL^{A1}-anti-PL^{A1} complexes binding to platelets. These results, however, are in conflict with the general concept that eluates of cell-associated immune complexes are nonreactive whereas cell-associated antibodies will demonstrate reactivity.[16]

Morrison and Mollison have proposed that a second antibody with autoantibody reactivity is also produced in PTP which reacts with PL^{A1} negative platelets.[3] In 3 patients we studied, autoreactivity was not found using acute plasma testing against autologous platelets following recovery.[17] Slichter also reported one patient in whom autoantibodies were not found.[18]

The third proposed mechanism of PL^{A1} antigenic material binding to PL^{A1} (−) platelets with subsequent destruction by the alloantibody is consistent with reported properties of platelets. Platelets may absorb many substances including proteins, blood group antigens, and HLA antigens.[5-7] Therefore, it is possible that PL^{A1} antigen may absorb to PL^{A1} (−) platelets if the antigen is transfused to a patient. To support this hypothesis, PL^{A1} antigen must be present in soluble form in stored blood.

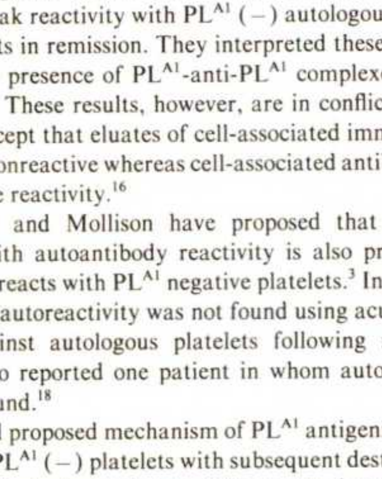

Fig 1. Lane A contains lysate from PL^{A1} (−) platelets first incubated with plasma from a PL^{A1} (+) donor; Lane B contains a lysate of PL^{A1} (−) platelets incubated with plasma from PL^{A1} (−) donor; Lane C contains a lysate of PL^{A1} (−) donor platelets; Lane D contains a lysate of PL^{A1} (+) platelets. The arrow identifies PL^{A1} reactivity. All lanes were immunoblotted using anti-PL^{A1}serum.

It is known that few intact platelets remain after 24 to 48 hours of storage at 4 °C. Therefore, antigenic material may exist as particulate material and possibly soluble forms. Despite meticulous blood collection, platelet deterioration occurs with storage. Ultrastructural alterations are consistent with loss of surface membrane integrity.[19] Various investigators have also described loss of platelet glycoproteins from the surface of stored platelets resulting from hydrolysis or protease activity.[20] These studies support the possibility that antigenic material may exist in stored plasma.

Previous attempts by Shulman and coworkers to detect a soluble form of PL^{A1} using a complement fixation technique were unsuccessful.[1] These investigators could detect large amounts of particulate PL^{A1} antigen in plasma but this material was removed with filtration. Similar experiments by Aster using a ^{51}Cr release assay also demonstrated PL^{A1} reactive material in serum which was removed by centrifugation.[21] More recently, Warejcka and coworkers have demonstrated PL^{A1} reactivity that did not sediment at 100,000 × g for 60 minutes.[22]

Our results show that following ultracentrifugation, PL^{A1} antigenic material is present in a form that can convert PL^{A1} negative platelets to a PL^{A1} positivity. In addition, using an inhibition assay, PL^{A1} antigen is present in ultracentrifuged plasma from all units of stored blood that were examined. The Western blotting experiments provide further verification that PL^{A1} antigenic material can be found in plasma from a PL^{A1} positive donor.

PL^{A1} antigen has been shown to be present on glycoprotein IIIa.[22] The molecular weight has been estimated to be 95,000 ± 10,000 daltons.[23] Although the converted platelets appear to have weaker reactivity than the PL^{A1}(+) control, they clearly acquired antigenic material of this molecular weight from the PL^{A1}(+) plasma.

Our studies suggest the following possible mechanism for posttransfusion purpura. PL^{A1} antigenic material from stored blood can absorb to PL^{A1} negative platelets. Upon transfusion, this material may bind to the patient's PL^{A1} negative platelets. With the formation of anti-PL^{A1} following an anamnestic immune response, the patient's platelets are destroyed leading to thrombocytopenia.

This explanation based on our in vitro experiments must also be evaluated in terms of clinical observations. Some patients with PTP may have thrombocytopenia for 4 weeks.

This would imply that sufficient antigen must be present for the duration of the thrombocytopenia. Intuitively, one would not expect prolonged survival of a transfused antigen unless there is a recycling process. Alternatively it is conceivable that, following the development of thrombocytopenia, the relative amount of antigen to the smaller number of platelets being produced is sufficient to maintain the thrombocytopenia. Shulman first proposed this theory to explain the prolonged course of thrombocytopenia.[4] To verify if this is the situation, further studies are needed to determine if PL^{A1} antigenic material is detectable in the patient's serum. Successful attempts at measuring circulating antigen have not been reported in patients with PTP.

Also, careful clinical observations are needed to determine if, in fact, the prolonged thrombocytopenia seen in some patients is related to PTP etiologic factors versus complicating clinical problems that impair thrombopoiesis.

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Increased Infection Mortality and Decreased Neutrophil Migration Due to a Component of an Artificial Blood Substitute

By Thomas A. Lane and G.E. Lamkin

We previously showed that an artificial blood substitute containing perfluorocarbons, Fluosol-DA, inhibited both neutrophil migration and adherence, due to its detergent component, Pluronic F-68. The purpose of the studies we report here was to determine if Fluosol or Pluronic might also reduce in vivo neutrophil migration and impair host resistance to bacterial infection. We studied in vivo PMN migration by injecting mice intraperitoneally (IP) with glycogen, followed by intravenous (IV) infusion of saline, Fluosol, or Pluronic. Peritoneal lavage after eight hours showed a significant decrease in the accumulation of PMN in lavage fluids of animals given either Fluosol or Pluronic (control—.19 ± .03 × 10^6 PMN/mL, glycogen—1.35 ± .14; glycogen/Fluosol—0.63 ± .12; glycogen/Pluronic—0.69 ± .07). We ascertained the effect of Fluosol and Pluronic on infection mortality by injecting mice IV with saline, Fluosol, or Pluronic, followed by a quantity of *E coli* (0.6 × 10^7) IP shown in preliminary studies to kill 20% to 50% of the mice in 24 hours. The 24-hour mortality was 14/45-saline, 24/32-Fluosol ($\chi^2 = 17.1$; $P < .001$) and 17/23 – Pluronic ($\chi = 11.2$; $P < .001$). Neither Fluosol nor Pluronic caused mortality without *E coli*. The increase in infection mortality occurred when Fluosol was given either two hours before, or simultaneously with *E coli*, but only with the simultaneous administration of bacteria and Pluronic. Pluronic did not alter reticuloendothelial system (RES) clearance function. These studies indicate that, in an animal model, Fluosol-DA, due to its detergent component Pluronic F-68, impaired neutrophil delivery to an inflammatory locus, and resulted in an increased rate of infection mortality. Since Pluronic did not result in RES blockade, but did impair the delivery of PMN to an inflammatory locus, our results suggest that the latter effect is responsible for the increase in infection mortality.

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THE function of certain candidate artificial blood substitutes (ABS) is based on oxygen transport by perfluorocarbons.[1-3] Since perfluorocarbons are immiscible in blood or water, they must be packaged in the form of an emulsion.[1] An extensively investigated ABS, Fluosol-DA, employed an emulsifier consisting of phosphatidyl choline and the nonionic surfactant, Pluronic F-68.[1] Pluronics are block copolymers consisting of polyoxypropylene (PP) and polyoxyethylene (PE) which have varying molecular weights and ratios of hydrophobe (PP) to hydrophil (PE).[4] Pluronic F-68 is an 8,500 Dalton copolymer with 80% PP.[4] ABS containing Pluronic F-68 may have adverse effects on phagocyte function.[5-8] Virmani and coworkers initially showed that incubation of granulocytes (PMN) with perfluorocarbon emulsions was accompanied by impairment of PMN phagocytosis and altered morphology.[6] These investigators subsequently reported alterations in several PMN functions, including ingestion, superoxide anion generation, adherence, aggregation, and chemotaxis, after incubation of PMN with Fluosol-DA.[7] We showed that Fluosol-DA inhibited neutrophil chemotaxis and adherence, and that this effect was entirely due to the surfactant component, Pluronic F-68.[5] We also showed that the serum of patients, to whom Fluosol-DA had been administered, was inhibitory of neutrophil chemotaxis and adhesion.[5] Normal chemotactic function of neutrophils is vital to host antimicrobial defenses, as evidenced by the increased risk of infection in patients with clinical chemotaxis defects.[9] Since candidate artificial blood substitutes containing Pluronic F-68, when used in clinically relevant doses in humans, are potent inhibitors of chemotaxis and perhaps other granulocyte functions,[6,7] it is possible that administration of such substances, either for hemotherapy or other purposes,[3] may impair host resistance to bacterial infection. We report here the extension of our preliminary study[10] regarding the effects of infusion of Fluosol-DA and Pluronic F-68 on migration of neutrophils into an inflammatory locus in vivo, and on overall host resistance to infection in an animal model.

METHODS

Neutrophil migration into an inflammatory locus was examined by injecting Swiss white mice (Simonson Laboratories, Gilroy, Calif) weighing 28 to 32 g intraperitoneally (IP) with 0.5 mL of 10% oyster glycogen (Sigma Chemical Company, St. Louis, Mo)[11] followed by intravenous (IV) injection of 1.6 mL of either (*a*) 0.85% sterile saline (controls), (*b*) Fluosol-DA (Alpha Therapeutics, Irvine, Calif) prepared according to the manufacturer's instructions, or (*c*) 27 mg/mL of a Pluronic F-68 solution in 0.85% sterile saline (BASF Wyandotte, Wyandotte, Mich). This concentration of Pluronic is equivalent to that found in Fluosol-DA.[1] Just prior to infusion, the pH of all solutions was adjusted to 7.4, followed by millipore (0.2 mm) filtration. Neutrophil accumulation in the peritoneal cavity was quantitated by lavaging the peritoneal cavity with 10 mL of sterile saline followed by white blood cell counting (Coulter Model ZF, Coulter Electronics, Hialeah, Fla). Differential cell counts were made on Wright's stained smears.

Mortality due to infection was examined using a previously described model of *Escherichia coli* peritonitis in mice.[12,13] A local strain of *E coli* which is resistant to killing by serum alone (VAMC-06, kindly supplied by J. Fierer, MD, Chief Microbiology Lab, VA Medical Center, San Diego, Calif) was grown in trypticase soy broth overnight, then 50 μl were grown for four hours in 5 mL fresh tissue culture medium (Hank's balanced salt solution). The bacteria were washed three times in saline, and resuspended in saline at 4 °C. Bacteria concentrations were ascertained by reading optical density at 610 nm and interpolation using standards of viable (colony-forming) bacteria. In five preliminary experiments, 4 to 6 groups of 4 mice each were injected IP with 0.3 mL of bacteria in concentrations ranging from 1 × 10^6 to 5 × 10^7/mL. In spite of careful attention to

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consistent technique, there was variation in mortality from one experiment to the next. We found that 24-hour mortality was 34 ± 12% after injection of 0.5 ± 0.18 (× 10^7) bacteria. In subsequent experiments the washed bacteria were adjusted to a concentration of 2 × 10^7/mL with saline. Mice received 0.3 mL of *E coli* (total dose = 0.6 × 10^7 bacteria) IP followed by IV injection of 1.6 mL of (*a*) 0.85% sterile saline, (*b*) Fluosol-DA, or (*c*) 27 mg/mL Pluronic F-68 in saline. Mortality was evaluated after 24 hours. In a preliminary peritonitis experiment, injection of 0.3 mL of bacteria IP, followed by infusion of 1.6 mL of the "annex solutions" used to reconstitute the Fluosol-DA resulted in a 24-hour mortality rate similar to that after infusion of 1.6 mL of 0.85% sterile saline (30% mortality, n = 10 each); hence, saline was used in the remaining experiments as the control infusion, and as the solvent for Pluronic F-68. The effect of the time of administration of Fluosol-DA and Pluronic F-68 on *E coli* periotonitis mortality was examined in three (Pluronic F-68) or four (Fluosol-DA) groups of animals. Group 1 received 1.6 mL IV of saline or Fluosol-DA, or Pluronic F-68 (27 mg/mL), followed in two hours by 0.3 mL of *E. coli* (2 × 10^7/mL) IP. Group 2 received saline, or Fluosol DA or Pluronic, and IP *E coli* at the same time, and Groups 3 and 4 received IP *E coli* followed in two or four hours by IV saline, or Fluosol-DA, or Pluronic F-68 (see text). Mortality was evaluated 24 hours after injection of *E coli*.

Reticuloendothelial system clearance of colloidal carbon (CC) was measured in 2.5 kg New Zealand white rabbits as previously described with minor modification.[14] Briefly, each animal was infused with an approximately 1/2 blood volume dose (55 mL/kg ÷ 2 = 27.5 mL/kg) of either 0.85% sterile saline or Pluronic (27 mg/mL) in 0.85% sterile saline, followed in three hours by infusion of 2 mL/kg of CC. Blood samples were taken prior to infusion of CC and 2, 4, 8, 12, 16, 20, and 30 minutes after CC. 50 μL blood samples were lysed in 700 μL of 1% Na_2CO_3, and read on a spectrophotometer at 695 nm with a red filter. Results were recorded as the difference in optical density (OD) of the sample compared to the preinfusion sample. Mixture of known amounts of CC with blood resulted in linear changes in OD over the range of values reported here; hence, the half disappearance time (T/2) of CC was determined graphically in each experimental subject.

The effect of Pluronics F-68 and F-108 (BASF-Wyandotte) on human neutrophil adherence to nylon wool columns and chemotaxis toward F-Met-Leu-Phe (10^{-8} mol/L, Sigma) were examined as previously described.[5,15-17]

Pluronics F-68 and F-108 (BASF-Wyandotte) have a similar ratio (1/4) of polyoxyethylene (hydrophil) to polyoxypropylene (hydrophobe) but different molecular weights (F-68 = 8,500 Daltons; F-108 = 14,700 Daltons). Of the many pluronics available, we chose F-108 for these studies because preliminary experiments in our laboratory revealed that F-108 was also a potent inhibitor of PMN chemotaxis.[18]

Data is expressed as the mean ± SE; statistical evaluations employed χ^2 analysis or the Student's *t* test.[19]

RESULTS

Preliminary experiments revealed that maximum neutrophil (PMN) influx into the peritoneal cavity of control mice given IP glycogen occurred between eight to 12 hours after installation of IP glycogen; consequently, lavage was performed eight hours after glycogen installation in the following experiments. As shown in Table 1, glycogen induced a nearly 7-fold increase in the number of PMN recoverable by peritoneal lavage in mice. The glycogen-induced influx of PMN was significantly blunted by simultaneous infusion of either Fluosol-DA or Pluronic F-68.

Table 1. Effect of Fluosol DA and Pluronic F-68 on Intraperitoneal Accumulation of Neutrophils

Agents (IP/IV)	PMN Delivery × 10^6/mL	n
Saline/saline	0.19 ± .03	20
Glycogen/saline	1.35 ± .14*	14
Glycogen/Fluosol-DA	0.63 ± .12†	10
Glycogen/Pluronic F-68	0.69 ± .07†	10

*$P < .001$ *v* saline/saline (two-tailed Student's *t* test).

†$P < .001$ *v* glycogen/saline (two-tailed Student's *t* test).

Having established that Fluosol-DA and its detergent component Pluronic F-68 both decreased the inflammatory response of PMN in the peritoneal cavity, we examined the effect of Fluosol-DA and Pluronic F-68 on mortality, due to *E coli* peritonitis, in mice. In two experiments, summarized in Table 2, infusion of a 1/2 blood volume of either Fluosol-DA or Pluronic F-68 (27 mg/mL) at the time of bacterial injection IP resulted in significantly increased mortality after 24 hours, compared to the control animals who were given only saline. Infusion of a 1/2 blood volume of either Fluosol-DA or Pluronic F-68 in animals given no *E. coli* did not cause mortality (n = 10 each). We also determined that infusion of a 1/2 blood volume of Pluronic (27 mg/mL) did not alter the rate of RES clearance of colloidal carbon in rabbits (mean T/2 = 5.0 min, n = 2) compared to control rabbits (mean T/2 = 5.5 min, n = 3).

The effect of time of administration of Fluosol-DA or Pluronic F-68 on *E coli* peritonitis mortality was studied. As shown in Table 3, in one experiment, Fluosol-DA infusion was associated with increased mortality when given prior to or simultaneously with bacteria. Animals injected with saline had insignificant differences in mortality at all time points. In two separate experiments, infusion of Pluronic F-68 was associated with increased mortality only when administered simultaneously with *E coli*.

In additional experiments, we compared the effect of Pluronics F-68 and F-108 on neutrophil chemotaxis and adherence in vitro. As shown in Fig 1, Pluronic F-108 was a more potent inhibitor of neutrophil function than Pluronic F-68. In three such studies the mean concentrations of Pluronic F-108 required for 50% inhibition (ID_{50}) of neutrophil chemotaxis and adhesion were 2 ± 1 mmol/L and 21 ± 9 mmol/L respectively. The respective ID_{50} values for Pluronic F-68 were 34 ± 3 mmol/L and 203 ± 24 mmol/L. Finally we found that infusion of Pluronic F-108 (1/2 blood volume of 4.8 mg/mL Pluronic F-108—see Methods) also significantly increased mortality due to *E coli* peritonitis in mice (saline

Table 2. Effect of Fluosol DA and Pluronic F-68 on *E coli* Peritonitis Mortality

Agent	Alive	Dead (%)	χ^2	*P*†
Saline	31*	14(32)	—	—
Fluosol-DA	8	24(75)	17.1	<.001
Pluronic F-68	6	17(74)	11.2	<.001

*Numbers refer to number of animals remaining alive or dead, 24 hours after injection of *E coli* (see Methods).

†Two-tailed test.

Table 3. Effect of Time of Infusion on Fluosol DA and Pluronic F-68–Induced Augmentation of *E coli* Peritonitis Mortality

	Mortality (%)			
Time of Infusion	Saline	Fluosol-DA	Saline	Pluronic
−2 h	0/8 (0)	5/8 (62)*	6/13 (46)	7/13 (54)
0	2/7 (29)	6/8 (75)*	7/13 (54)	11/13 (85)*
+2 h	2/8 (25)	4/8 (50)	ND	ND
+4 h	2/7 (29)	3/8 (38)	3/10 (30)	4/10 (40)

Time of infusion refers to number of hours by which saline, Fluosol-DA, or Pluronic F-68 IV infusion preceeded or followed IP injection of *E. coli.* Mortality refers to number of mice dead at 24 hours/total number of mice. ND, not done.

*$P < .05$ (single tailed significance test), compared to saline group.

control—1/10 dead in 24 h; Pluronic F-108—5/10 dead, $P = .05$).

DISCUSSION

An ideal artificial blood substitute should not only carry oxygen and support multiple transport functions, but should also have no deleterious effects on other critical functions that are facilitated by the vascular system. Prevention of microbial infection is one such function carried out by the vasculature. It has been reported that at least one extensively investigated experimental artificial blood substitute (Fluosol-DA) did not interfere with the immune system[1]; however, we and others demonstrated that Fluosol-DA profoundly impaired the ability of neutrophils to migrate and to adhere.[5-7] We showed that these effects occurred after administration of clinically relevant doses in humans.[5] We further showed that, in serum-free media, Fluosol-DA did not disturb other cell functions, that the effect of Fluosol-DA on migration and adherence extended to other cell types (eg, macrophages) and nonhuman phagocytes, that these effects were rapidly reversible both in vitro and in vivo, and that they were due entirely to the detergent component of the artificial blood substitute, Pluronic F-68.[5]

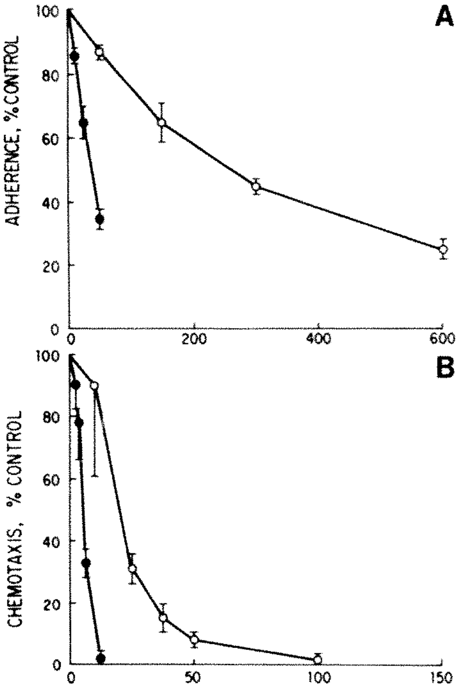

Fig 1. Effect of Pluronics F-68 and F-108 on granulocyte adherence (A) and chemotaxis (B). Data points represent mean ± SE of triplicate determinations in a typical experiment (see text). ○, F-68; ●, F-108.

The above findings raised the possibility that any candidate blood substitute containing Pluronic F-68, or other pharmacologic preparations containing Pluronic F-68, or Pluronic F-68 alone, might have a similar deleterious effect on neutrophil migration in vivo. If so, we hypothesized that animals (or potentially humans) given these substances might be particularly susceptible to infection. We report here that infusion of Fluosol-DA, or a corresponding amount of Pluronic F-68 alone, used in doses similar to that which have been used in both animals and humans,[2-5] significantly impaired the accumulation of PMN in the peritoneum after a standard inflammatory challenge. We further demonstrated that animals pretreated with Fluosol-DA or Pluronic F-68 suffered increased mortality due to experimental peritonitis. The association between Pluronic-induced impairment in PMN migration in vivo and increased infection mortality strongly supports a causal relationship between the two. Other investigators have reported that perfluorocarbon emulsions containing pluronic may interfere with other PMN functions in addition to migration.[6,7] This may also have contributed to the excess mortality in our experiments. Studies in our laboratory did not reveal alterations in other PMN functions.[5] These differences may have been due to variations in methodology, as previously discussed.[5] It has been reported that Fluosol-DA also partially blocks the RES.[14,20] Our finding that Pluronic F-68 infusion did not alter RES clearance indicates that RES blockage cannot explain the effect of Pluronic F-68 on infection mortality observed in these studies.

The increased infection mortality we observed after infusion of Pluronic occurred only when it was infused simultaneously with bacteria injection. In a separate experiment, infusion of Fluosol-DA increased infection mortality even when administered prior to *E. coli.* This difference may be due to the longer circulating half-life of Pluronic F-68 when given in the form of the Fluosol-DA emulsion ($T/2 = 5.7$ h) than when given free in solution ($T/2 = 1.8$ h).[1] Direct comparison of the effect on mortality of infusion of Pluronic *v* Fluosol two hours prior to bacteria injection is mitigated by the marked difference in mortality of the two control groups. The reason for the failure of Pluronic F-68 or Fluosol-DA to augment infection mortality when given two to four hours after bacteria is unclear but is compatible with earlier observations that the outcome of a bacterial infection is determined within the first few hours of onset.[21]

Pluronic F-68 is but one of many nonionic surfactants that are available for use in the food, drug, and cosmetic industry.[4,22] We found that Pluronic F-108 was a more potent inhibitor of PMN function than F-68. Pluronic F-108 also increased infection mortality in mice. Thus the adverse effects of Pluronic F-68 on PMN function are not unique to this polymer, but may be a property common to most or all such agents. Further studies in our laboratory on additional Pluronics support this view.[18]

These studies have potential clinical implications. First, they suggest that the components of future candidate artificial blood substitutes should be carefully studied for inhibition of phagocyte function. Second, although Fluosol-DA is no longer undergoing trials as a blood substitute, it is currently being investigated in humans as an ultrasound imaging agent[23] and for a wide variety of conditions associated with tissue ischemia.[3] We are aware of no reports of increased infection mortality in humans to whom Fluosol-DA has been administered, and we do not advise discontinuation of the investigation of perfluorocarbon emulsions. However, our current studies, and a recent report of increased infection mortality after Fluosol exchange transfusion in an animal model similar to ours,[24] support the view that patients who are infused with Pluronic-containing substances should be carefully observed. Finally, these studies suggest that (1) Pluronic F-68 may serve as a useful probe of neutrophil chemotactic function both in vitro and in vivo, and (2) this or a similar agent may potentially be a useful therapeutic agent to specifically and reversibly alter PMN function. Preliminary studies of the effect of Pluronic on a neutrophil-dependent model of acute lung injury support this hypothesis.[25]

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Does the Conformation of Adsorbed Fibrinogen Dictate Platelet Interactions With Artificial Surfaces?

By Jack N. Lindon, Gerald McManama, Leslie Kushner, Edward W. Merrill, and Edwin W. Salzman

Platelet activation by polymer surfaces is thought to require preliminary adsorption of fibrinogen and perhaps changes in fibrinogen conformation. We measured fibrinogen adsorption by a series of polymers by two methods, using either ^{125}I-labeled fibrinogen or ^{125}I-labeled antifibrinogen antibodies, and correlated the results with platelet reactivity (retention and secretion) in columns of beads coated with the polymers. For polyalkyl methacrylates with 1 to 4 carbon side chains, platelet reactivity varied directly with increasing length of the alkyl side chain and with the quantity of bound fibrinogen recognizable by antifibrinogen antibody but not with the total quantity of fibrinogen adsorbed. The same pattern of results was seen with five antibody preparations, including affinity-purified Fab fragments against the D or E domain of fibrinogen. Tests of platelet retention and fibrinogen binding to four polyalkyl acrylates and to three unrelated polymers (polystyrene, polymethyl methacrylate, and a polyether polyurethane) indicated that platelet retention correlated positively with both total fibrinogen binding and with the amount of antibody-recognizable fibrinogen bound. Drugs that block platelet aggregation, but not adhesion, did not alter the hierarchy of platelet retention to the polyalkyl methacrylates. These data suggest that, contrary to previous views, platelet adhesion to artificial surfaces increases with increasing surface coverage of adsorbed fibrinogen if the bound fibrinogen maintains a conformation such that its functional domains remain recognizable by antibody probes.

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WHEN a synthetic polymer such as a prosthetic arterial graft or dialysis membrane is exposed to blood, the interactions of platelets and other blood cells with the artificial surface are preceded by formation of a layer of adsorbed plasma components composed primarily of proteins,[1,2] since the high concentration and diffusivity of plasma proteins, compared with the much larger blood cells, bring the proteins to the surface first. Interactions of plasma proteins in the fluid phase with the adsorbed protein film can also lead to activation of the coagulation, complement, and fibrinolytic systems. The composition of this protein film and the molecular configurations of its constitutents, as influenced by the nature of the underlying polymer surface, are, therfore, of particular interest.

Packham et al[3] and Zucker and Vroman[4] reported almost 20 years ago that fibrinogen potentiates the adhesion of platelets to glass surfaces. Notwithstanding the more recently described participation of other "adhesive proteins" (eg, factor VIII:VWF and fibronectin) in adhesion of platelets to subendothelial connective tissue,[5] reports from numerous laboratories have shown that for many surfaces the adsorption of fibrinogen rather than other plasma proteins correlates with the ability of the surfaces to promote platelet adhesion.[6-8] Many surfaces when exposed to mixtures of purified proteins or to whole plasma preferentially adsorb fibrinogen,[9-11] sometimes with subsequent desorption, which Vroman and coworkers[12,13] have reported to require high molecular weight kininogen. It has been suggested[14,15] that changes in the conformation of adsorbed fibrinogen molecules induced by interaction with the surface may lead to their recognition by platelets as "foreign" and may be responsible for platelet adhesion and spreading on the surface, and ultimately to secretion of the contents of storage granules and aggregation of additional platelets.

Experiments presented here were designed to test the hypothesis that variations in platelet interaction with surfaces exposed to whole blood correlate with the extent of fibrinogen adsorption. As a model system, we studied a series of closely related polymers—the polyalkyl methacrylates (Fig 1)—which consist of linear carbon backbones on which every second carbon possesses both a methyl group and a carboxylic acid group in ester linkage with an alkyl alcohol (eg, polybutyl methacrylate contains carboxylic acid groups complexed by ester linkage to n-butyl alcohol). The length of the alkyl side chains determines many of the polymers' physical and chemical properties.

We have previously tested platelet reactivity of the polyalkyl methacrylates using an in vitro system in which whole blood, maintained at 37 °C, was passed through polymer-coated glass bead columns, and platelet retention by the columns and secretion of ^{14}C-serotonin and beta thromboglobulin were measured.[16] Platelet reactivity (ie, platelet retention on the columns due to adhesion to the surfaces and platelet aggregation and secretion of platelet contents) was found to increase in parallel with the length of the alkyl side chain. Platelet retention in the columns varied from 30% for polymethyl methacrylate (PMMA) to approximately 70% for polybutyl methacrylate (PBMA). Secretion of beta thromboglobulin by platelets exposed to these polymers varied from 2 μg/mL for PMMA to 5 μg/mL for PBMA; serotonin secretion paralleled beta thromboglobulin secretion. We now report that these polymers do not vary in their capacity to adsorb fibrinogen from a solution of the purified protein or from whole blood plasma, contrary to the expected result, but that the platelet reactivity of the polyalkyl methacrylates appears to correlate with their ability to bind fibrinogen in its "native"or "unaltered" conformation (defined as the ability of the fibrinogen molecule to be recognized by antifibrinogen antibodies).

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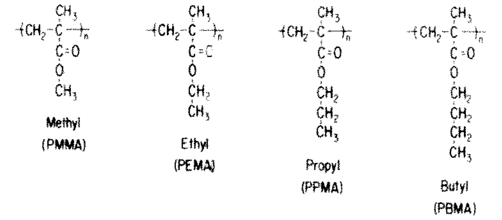

Fig 1. Structure of polyalkyl methacrylate polymers.

MATERIALS AND METHODS

Polymers. Polyalkyl methacrylates were synthesized by free radical polymerization initiated by exposing alkyl methacrylate monomers to high doses (~10 Mev) of ionizing radiation using a Van de Graff accelerator. The monomers (methyl, ethyl, n-propyl, and n-butyl methacrylate; Polysciences, Warrington, Pa) were placed in 20 mL glass vials and irradiated in an argon environment. After polymerization was allowed to proceed for several hours at room temperature, the polymers were separated from unreacted monomer by precipitating in methyl alcohol (Fisher Chemical Co, Boston, Mass) and redissolving in chloroform (Fisher Chem Co). Polymerized polyalkyl acrylates were purchased from Polysciences and were further purified by dissolving in chloroform and reprecipitating in methyl alcohol. Polystyrene (mol wt 300,000; Pressure Chem Co, Pittsburgh, Pa) and Biomer (lot no. BSR0I7A; Ethicon, Inc, Somerville, NJ) were used as received from the manufacturers. Polymers were dissolved in chloroform (20 mg/mL) for use in coating glass bead columns and glass cover slips.

Fibrinogen. Fibrinogen was purified from human plasma using a modification of the glycine:lysine precipitation technique described by Wang.[17] An additional step was added: the fibrinogen preparation was passed through a gelatin-Sepharose column to remove contaminating fibronectin. The resultant fibrinogen was >95% clottable by thrombin and showed, on SDS-PAGE, less than 1% contamination by either fibrinoectin or von Willebrand factor. Purified fibrinogen stocks were adjusted to 10 mg/mL with 0.3 mol/L NaCl and stored in aliquots at −60 °C.

Fibrinogen was labeled with ^{125}I using the "Iodogen"(1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril: Pierce Chemical Co, Rockford, Ill) technique.[18,19] Iodogen is an oxidizing agent, virtually insoluble in water, which, when coated on the wall of a glass vessel, catalyzes the iodination of the tyrosine moieties of protein in the presence of ^{125}I.[18] The inner surfaces of glass vials (12 mm × 45 mm) were coated with 10 μg of Iodogen by adding 0.2 mL of a 50 μg/mL solution of Iodogen in chloroform (Spectroscopic grade; Fisher Chemical Co) and evaporating to dryness at 37 °C. Phosphate-buffered saline (PBS: 125 mmol/L NaCl, 25 mmol/L NaH_2PO_4, pH 7.4), 150 μL, and 100 μg of purified fibrinogen were placed in an Iodogen-coated vial and 1 mCi of ^{125}I (Amersham, Inc, Arlington Heights, Ill) was added. The reaction was allowed to proceed, at 22 °C, with intermittent mixing for 10 minutes. The mixture was then chromatographed on a 0.6 cm × 17 cm G-25 Sephadex column (Pharmacia, Inc, Piscataway, NJ) which previously had been conditioned with 50 mL of a 100 μg/mL fibrinogen solution and then washed with 50 mL of PBS. The labeled fibrinogen was eluted with PBS. Elution patterns typically showed two peaks, one at the void volume containing labeled fibrinogen and a second at the column volume containing free ^{125}I, with >70% of the ^{125}I eluting with the fibrinogen. Labeled fibrinogen was stored at −60 °C in 25 μL aliquots and was used within 1 week of preparation. Isotope dilution experiments were performed on each batch of labeled fibrinogen and indicated that the assays of fibrinogen binding to the polymers were independent of the specific activities of the fibrinogen solutions employed.

Antibodies. Two commercial antihuman fibrinogen antibodies (IgG fractions; Atlantic Antibodies, Scarborough, Me and U.S. Biochemical Corp, Cleveland, Oh) and antihuman IgG antibody (IgG fraction; Sigma Chem Co, St. Louis, Mo) were used without further purification. In addition, Fab fragments of affinity purified antifibrinogen, anti-domain D and anti-domain E antibodies (kindly supplied by Drs Jacek Hawiger and Marek Kloczewiak) were prepared as previously described.[20] Briefly, serum from rabbits immunized against purified human fibrinogen was affinity purified using a fibrinogen-Sepharose column. The antifibrinogen antibodies were then passed through D-domain-Sepharose or E-domain-Sepharose affinity columns, and the anti-D-domain and anti-E-domain antibodies were eluted with 0.2 mol/L glycine, pH 2.4. Finally, Fab fragments of each of these three antibody preparations were prepared by papain digestion.

Antibodies were labeled with ^{125}I using the "Iodogen" technique described above. After labeling, antibodies were separated from unbound ^{125}I by chromatography on G-25 Sephadex columns preconditioned with PBS.

Fibrinogen adsorption. Fibrinogen binding to polymer-coated cover slips was measured using two different techniques. Total fibrinogen binding was assayed using ^{125}I-labeled fibrinogen. Binding of ^{125}I-labeled antifibrinogen antibody to cover slips that had been previously exposed to fibrinogen was also measured to determine the amount of antibody-recognizable fibrinogen on the polymer surfaces. Glass cover slips (1.2 cm diameter) were coated with polymer in a laminar flow hood by grasping with stainless steel forceps, dipping in a 2% polymer solution, and drying in the hood for 20 minutes.

For measurement of total fibrinogen binding, cover slips were immersed in a solution of purified fibrinogen containing ^{125}I-fibrinogen (concentration, 250 μg/mL; specific activity, ~1000 cpm/μg) or in whole or diluted plasma spiked with ^{125}I-fibrinogen (specific activity, ~3000 cpm/ng) and submerged for up to one hour under static conditions. The cover slips were then removed and washed for 15 seconds in PBS and the bound radioactivity was counted. For measurement of fibrinogen binding using labeled antifibrinogen antibodies (Fg-Ab*), the cover slips, after exposure either to purified fibrinogen (250 μg/mL) or to whole or diluted plasma, were washed in PBS and placed in 15 mm × 75 mm test tubes containing 0.5 mL of a solution containing bovine serum albumin (3 mg/mL; Sigma Chem Co) and Fg-Ab*. The slips were incubated for one hour, with continuous shaking, and were then washed with PBS and counted.

Platelet retention testing. Protocols for platelet retention testing in polymer-coated glass bead columns have been described in detail.[16,21] Briefly, polyethylene columns (0.8 cm × 20.5 cm) were filled with acid-washed, 0.3 mm diameter glass beads (total surface area, ~200 cm^2) and coated with polymer by deposition from a 2% chloroform solution. Polymer-coated columns were washed with saline and then exposed to whole blood (maintained at 37 °C) at a flow rate of 0.25 mL/min. Platelet counts were determined for four 1 mL effluent fractions of undiluted blood and for the blood prior to column exposure, and the percentage of platelet retention was calculated from the ratio of the average platelet count in the effluent fractions to the whole blood platelet count.

Statistical analysis. Comparisons among data for different polymers were made using 3-way analysis of variance and the Duncan's multiple range test for grouped, parametric populations.[22] Data organization and analysis were performed on the *Prophet* system, a national computer resource sponsored by the Chemical/Biological Information Handling Program, National Institutes of Health.

RESULTS

Total fibrinogen binding. Fibrinogen adsorption was measured by dipping polymer-coated cover slips into buffered fibrinogen solutions or into diluted or undiluted blood plasma, all containing radiolabeled fibrinogen. One objection to the use of a "dipping" technique for measuring protein adsorption is that the dipped surface may become coated with denatured protein as it passes through the air-solution interface (the Langmuir-Blodgett effect[23,24]). Figure 2 illustrates the binding isotherms obtained when polybutyl methacrylate–coated coverslips were exposed for eight minutes to various concentrations of purified fibrinogen solutions labeled with ^{125}I-fibrinogen. Identical isotherms were obtained by dipping polymer-coated cover slips into fibrinogen solutions or by adding fibrinogen to buffer in which the coverslips were already immersed, indicating that denatured fibrinogen at the air-solution interface did not affect the measurement of fibrinogen binding in this system. Binding of fibrinogen to PBMA increased markedly as the fibrinogen concentration was increased to approximately 50 μg/mL. Above 50 μg/mL, binding increased only marginally with increasing fibrinogen concentration. However, a saturation plateau was not obtained even with fibrinogen concentrations as high as 2 mg/mL. Binding isotherms were similar for each of the polyalkyl methacrylates.

Measurement of the time course of ^{125}I-fibrinogen binding to the four polyalkyl methacrylates indicated that the bulk of the binding occurred within the first 30 seconds, with a slow, progressive increase in binding occurring thereafter (Fig 3A). Exposure of the polyalkyl methacrylate polymers to 250 μg/mL fibrinogen solutions for one hour resulted in the binding of approximately 0.3 μg/cm^2 and did not differ significantly among the four polymers ($P > 0.10$, $n = 9$). Fibrinogen adsorption in this range probably represents a monolayer of bound protein (see Discussion).

When the polyalkyl methacrylates were incubated in plasma for up to 60 minutes, a more gradual increase in binding occurred (Fig 3B), with total binding after 60 minutes approaching 0.2 μg/cm^2 for each of the methacrylates. However, Vroman et al[12] have shown that, for surfaces exposed to plasma, competition with other plasma proteins (especially high molecular weight kininogen) can quickly and markedly reduce the amount of fibrinogen bound. Brash et al[13] have shown that these early adsorption and desorption events occur more slowly and can be more readily examined if the plasma is diluted. When the polymers were exposed to 20% plasma (1 part plasma diluted with 4 parts PBS), fibrinogen binding was greatest at the earliest time point measured (15 seconds) and then decreased quickly to an apparent plateau at approximately 0.16 μg/cm^2 (Fig 3C). When the methacrylate polymers were exposed to 1% plasma, fibrinogen-binding kinetics showed a maximum at one to three minutes followed by a decrease in bound fibrinogen to an apparent plateau at less than 0.1 μg/cm^2(Fig 3D). For undiluted plasma and 20% plasma, the time course and amounts of fibrinogen binding were indistinguishable for the four methacrylate surfaces. For 1% plasma, the binding kinetics were similar for the four polymers and the levels of fibrinogen bound to the polyethyl, propyl, and butyl methacrylates were not significantly different ($P > 0.10$, $n = 3$). However, for incubations of up to eight minutes in 1% plasma, fibrinogen binding to polymethyl methacrylate was slightly, but significantly ($P < 0.05$, $n = 3$) less than to the other three polymers.

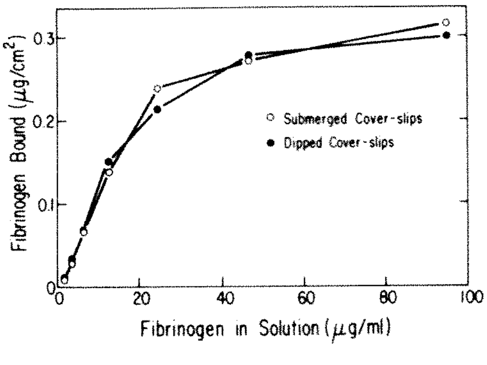

Fig 2. Binding isotherms of fibrinogen binding to polybutyl methacrylate. Polymer-coated cover slips were either dipped into ^{125}I-labeled fibrinogen solutions (●) or placed in buffers prior to the additon of ^{125}I-fibrinogen (O). Cover slips were incubated and processed according to protocols outlined in Materials and Methods.

Fibrinogen binding assayed with antifibrinogen antibodies. Surface-bound fibrinogen was also assessed using polyclonal antibodies raised against human fibrinogen and labeled with ^{125}I. Preliminary experiments (data not shown) indicated that binding of antifibrinogen antibodies to fibrinogen-coated polyalkyl methacrylates was essentially complete in one hour. Antifibrinogen antibody adsorption to polyalkyl methacrylates that had first been exposed to solutions of purified fibrinogen (250 μg/mL) for up to 60 minutes is shown in Fig 3E. Binding ranged from 1.26 ng/cm^2 for PMMA to 3.11 ng/cm^2 for PBMA, after the 60-minute exposure, and was significantly different for each of the polymers at each time point tested ($P < 0.05$, $n = 9$). These low levels of antibody binding were due to the low concentration of labeled antibody solution employed. When the antibody concentration was increased, antibody binding increased to levels similar to that of the bound fibrinogen and the differences in antibody binding to the four polymers were no longer observed (Table 1).

When the polyalkyl methacrylates were first exposed to plamsa for up to 60 minutes and then to a low concentration of antifibrinogen antibody (Fig 3F), antibody binding among the four methacrylates was not significantly different after preexposure to plasma for 8 or 60 minutes; but, after short preexposures (15 seconds, 1 minute, or 2.5 minutes), small but statistically significant differences in antibody binding to each of the four methacrylates ($P < 0.05$, $n = 14$) were observed. When 20% plasma was employed for preexposure (Fig 3G), the differences in antibody binding to the four methacrylates at the early time points were larger, but still disappeared after eight minutes of preexposure. When the polymers were preexposed to 1% plasma (Fig 3H), the subsequent differences in antibody binding were large and statistically significant ($P < 0.05$, $n = 6$) at all time points tested. When antifibrinogen antibody binding was assessed

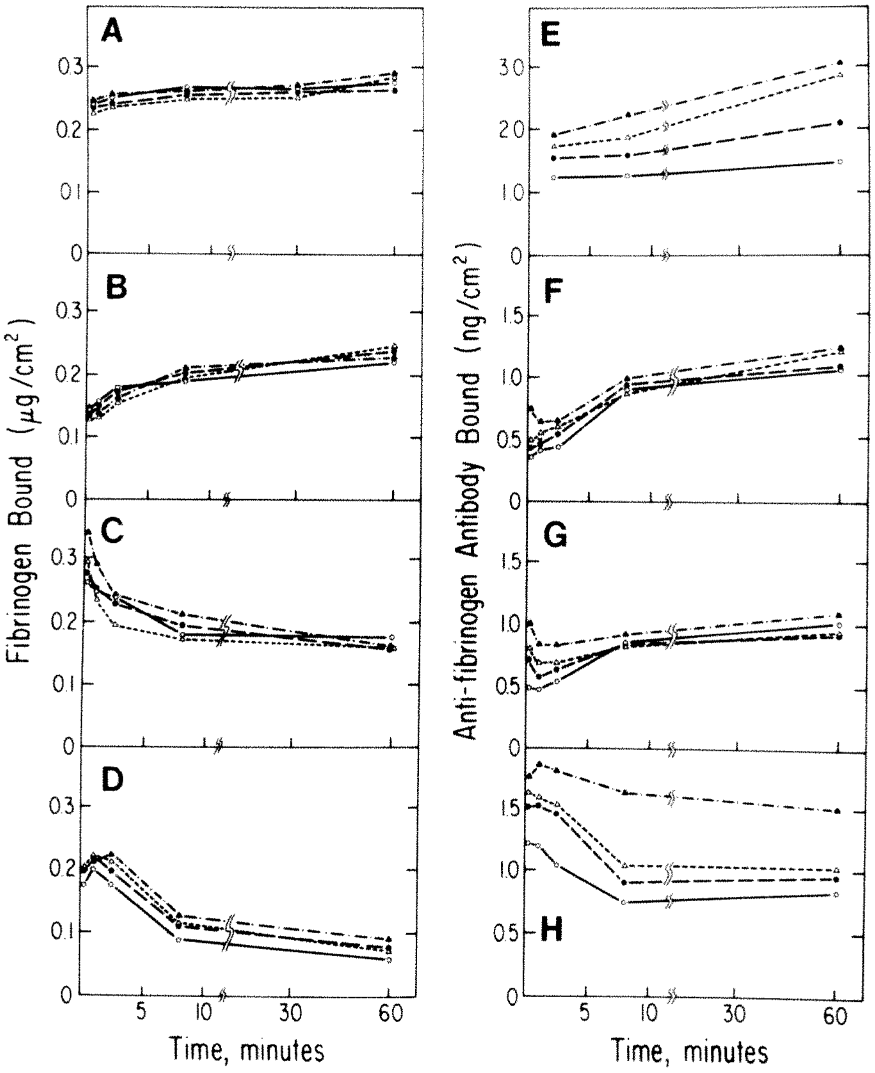

Fig 3. Fibrinogen binding and antifibrinogen antibody binding to polyalkyl methacrylates. Polymer-coated cover slips were exposed to solutions spiked with ^{125}I-labeled fibrinogen (A-D) or were exposed first to unlabeled solutions and then to ^{125}I-labeled antifibrinogen antibody (E-H) according to protocols outlined in Materials and Methods. (A) Purified fibrinogen solution (250 μg/mL). (B) Undiluted plasma. (C) 20% plasma. (D) 1% plasma. (E) Purified fibrinogen solution (250 μg/mL) followed by antifibrinogen antibody solution (300 ng/mL, ~800 cpm/ng). (F) Undiluted plasma followed by antifibrinogen antibody solution (100 ng/mL, ~8,000 cpm/ng). (G) 20% plasma followed by antifibrinogen antibody solution (100 ng/mL, ~8,000 cpm/ng). (H) 1% plasma followed by antifibrinogen antibody solution (100 ng/mL, ~8,000 cpm/ng).

concomitantly for methacrylates preexposed either to a fibrinogen solution or to plasma, comparable binding was observed. For example, preexposure of the polymers for one minute to either undiluted plasma, 1% plasma, or fibrinogen (250 μg/mL) followed by incubation with labeled antibody (100 ng/mL) led to antibody binding ranging from 0.58 to 0.81 ng/cm² for undiluted plasma, 0.87 to 2.6 ng/cm² for 1% plasma, and 1.5 to 2.2 ng/cm² for fibrinogen solution. As shown in Fig 4, there are statistically significant positive correlations between antifibrinogen antibody binding and both platelet retention (R = 0.984, $P < 0.02$) and beta thromboglobulin release (R = 0.978, $P < 0.03$) induced by the polyalkyl methacrylates. These results suggest that platelet reactivity on these polymer surfaces may be dictated by the amount of antibody-recognizable ("native") fibrinogen on the surface rather than by total fibrinogen adsorption.

Table 1. Binding of Antifibrinogen Antibody (Fg-Ab) to Polyalkyl Methacrylate Polymers Previously Exposed to Fibrinogen*

	Fg-Ab Bound (ng/cm²)			
Polymer	0.3 μg/mL†	1 μg/mL	10 μg/mL	200 μg/mL
PMMA	1.26 ± .12‡	5.7 ± .4	108 ± 5	250 ± 27
PEMA	2.09 ± .12	6.3 ± .3	102 ± 8	251 ± 13
PPMA	2.94 ± .05	8.1 ± .5	100 ± 7	234 ± 14
PBMA	3.07 ± .07	9.4 ± .8	105 ± 3	237 ± 17

*Polymers were exposed to a solution of purified fibrinogen (250 μg/mL) for one hour.

†Values shown are the concentrations of antifibrinogen antibody solutions to which polymers were exposed following initial fibrinogen exposure.

‡Data represent means ± standard deviations: for the 0.3 μg/mL column, n = 9; for the 1, 10, and 200 μg/mL columns, n = 3.

Differences in antifibrinogen antibody binding to polymer surfaces coated with nearly identical amounts of bound fibrinogen might result from variations in the orientation of the fibrinogen molecules on the different polymers or from variation in the extent of the conformational alterations induced in fibrinogen by the energy of adsorption. If the different methacrylates bind fibrinogen with no particular orientation but with the induction of progressively severe conformational alterations, then a variety of antibodies should show the same hierarchy of affinity for the surface-bound fibrinogen molecules. If, in the other hand, the different methacrylates do not induce conformational

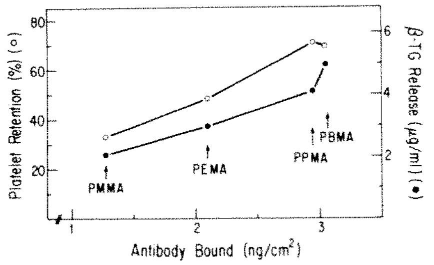

Fig 4. Correlation of antifibrinogen antibody binding with platelet activation by polyalkyl methacrylates. Polymer-coated cover slips were exposed first to fibrinogen and then to ^{125}I-labeled antibody according to the protocol outlined in Materials and Methods. Specific activity and concentration of the ^{125}I-labeled antibody preparations were ~800 cpm/ng and 300 ng/mL. Values for platelet retention and beta-thromboglobulin secretion were previously reported.[16] Platelet retention differed significantly ($P < 0.05$) among the four polymers except that no significant difference was obtained between retention values for PPMA and PBMA. Beta thromboglobulin release was significantly different ($P < 0.05$) for each of the four polymers.

changes but simply adsorb the fibrinogen with different orientations, then antibodies that recognize different parts of the fibrinogen molecule should show different hierarchies of antibody binding.

In order to test these two possibilities, experiments were conducted using three different antifibrinogen antibody preparations (two whole IgG fractions and one Fab fragment) and also Fab fragments of antibodies prepared against the D-domain (the terminal region) and the E-domain (the central region) of fibrinogen. Although the extent of the binding varied significantly from one antibody preparation to another, the rank order of antibody binding to the polyalkyl methacrylates was the same for each antibody (Fig 5). As a control, binding of an anti-IgG antibody preparation to the fibrinogen-coated surface was 10- to 100-fold less than that of the antifibrinogen antibodies and showed no material-to-material variation. For each of these polyclonal antibody preparations (even the anti-D and anti-E-region antibodies), binding to surface-bound fibrinogen molecules must be a complicated function of interactions within numerous epitope-antibody systems. However, these results suggest that the rank order of antifibrinogen antibody binding to the polyalkyl methacrylates was due to the degree to which each of these surfaces altered the conformation of bound fibrinogen molecules rather than to differences in the orientation of the bound fibrinogen molecules on the surfaces.

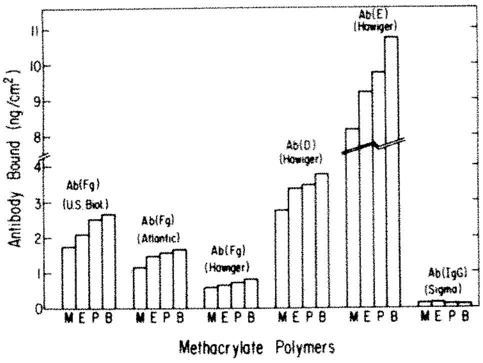

Fig 5. Binding of antifibrinogen antibodies to polyalkyl methacrylates exposed to fibrinogen. Two commercial antifibrinogen antibody IgG preparations (U.S. Biochemical and Atlantic Antibody), an antifibrinogen Fab fragment [Ab(Fg)], an anti-domain D Fab fragment [Ab(D)], an anti-domain E Fab fragment [Ab(E)], and an anti-IgG antibody [Ab(IgG)] were used. Polymer-coated cover slips were exposed first to fibrinogen and then to ^{125}I-labeled antibody preparations according to the protocols outlined in Materials and Methods. Specific activities and concentrations of each of the ^{125}I-labeled antibody preparations were ~1,000 cpm/ng and 200 ng/mL.

Platelet retention and fibrinogen binding by other polymers. In order to test the generality of the finding that platelet activation on polyalkyl methacrylates correlates with the binding of unaltered fibrinogen, platelet retention tests and fibrinogen binding assays were performed with two additional groups of polymers, the polyalkyl acrylates and a selected group of unrelated polymers. Polyalkyl acrylates have a structure similar to that of the polyalkyl methacrylates except for the replacement with hydrogen atoms of the methyl groups on the carbon backbone. A previous study[16] showed that platelet activation in glass bead columns coated with various polyalkyl acrylates increased as the length of the polymer side chain increased from one carbon (polymethyl acrylate, PMA) to four carbons (polybutyl acrylate, PBA). As shown in Fig 6A, variations in both total fibrinogen binding and antifibrinogen antibody binding correlated with the increase in platelet retention seen with the longer side chain acrylates.

Direct comparisons of platelet retention and fibrinogen binding were also made using two other polymers, polysty-

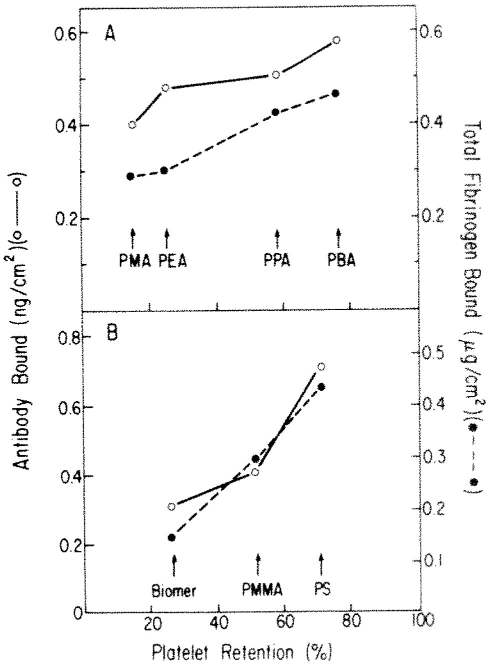

Fig 6. Correlation of antifibrinogen antibody binding and total fibrinogen binding with platelet retention by (A) four polyalkyl acrylates and (B) three other polymers. Binding protocols and platelet retention tests are described in Materials and Methods. Specific activity and concentration of the ^{125}I-labeled antibody preparations were ~10,000 cpm/ng and 20 ng/mL.

rene and a segmented polyether polyurethane (Biomer), which were compared with polymethyl methacrylate in the same experiment. Platelet retention ranged from 27% for Biomer to 52% for PMMA and 71% for polystyrene. With this group of polymers, as with the polyalkyl acrylates, variations in both total fibrinogen binding and antifibrinogen antibody binding correlated with the differences in platelet retention (Fig 6B). The apparent discrepancy in platelet retention values for PMMA in this study and in the previous study of polyalkyl methacrylates (Fig 4) is due to the fact that different blood donors were used for the two studies and that there may be substantial donor-to-donor variability in the platelet retention test.[21] For this reason, comparisons of platelet retention by different materials are only made for groups of materials tested simultaneously using the same blood donors.

Effect of platelet aggregation inhibitors on platelet retention. The results presented above indicate that the binding of "unaltered" fibrinogen to polymer surfaces correlates with platelet retention on the surfaces, even when the *total* amount of fibrinogen bound does not. However, platelet retention in polymer-coated glass bead columns results from both platelet adhesion to the surfaces and subsequent formation of platelet aggregates. In order to determine whether the binding of unaltered fibrinogen to artificial surfaces is involved in platelet adhesion, platelet retention on polyalkyl methacrylate-coated columns was studied using whole blood with or without a platelet aggregation inhibitor. In vitro addition of indomethacin, or aspirin ingestion two hours prior to blood donation, decreased the extent of platelet retention by the three polyalkyl methacrylates tested but did not change the rank order (Fig 7). Analysis of variance indicated that aspirin produced an insignificant drop ($P = 0.25$) in platelet retention and that indomethacin produced a significant drop ($P < 0.05$) in platelet retention that appeared, from examination of the bead columns by scanning electron microscopy,[21,25] to be due to the nearly complete elimination of platelet aggregates on the polymer surfaces. Data analysis by Duncan's multiple range test[22] showed significant differences ($P < 0.05$) among the polymers both before and after drug treatment. Thus, the correlation noted above between "native" fibrinogen binding and platelet *retention* also holds for platelet *adhesion* to the polyalkyl methacrylates. It is possible, therefore, that platelet adhesion to polymer surfaces is mediated by unaltered, surface-bound fibrinogen.

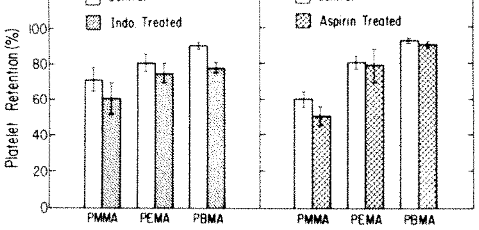

Fig 7. Effects of indomethacin or aspirin on platelet retention by polyalkyl methacrylate-coated glass bead columns. (A) Retention was measured using whole blood or whole blood to which indomethacin had been added (final concentration, 100 μmol/L). Test was repeated with 5 different donors. (B) Retention was measured before and two hours after ingestion of 650 mg of aspirin. Test was repeated with 5 different donors. Different groups of donors were used for each drug test.

DISCUSSION

The apparent requirement of platelet adhesion to surfaces for "native" adsorbed fibrinogen (ie, fibrinogen recognizable by antifibrinogen antibody and therefore presumably only minimally altered in conformation by adsorption onto the surface) is reminiscent of the essential role fibrinogen plays in platelet aggregation.[26-28] In plasma, fibrinogen does not induce platelet aggregation but acts as a cofactor, binding to platelets only after platelet stimulation by agonists such as ADP which induce the expression of glycoprotein receptors for fibrinogen on the platelet surface.[20,29] The bound fibrinogen may then act, possibly in concert with von Willebrand factor,[30] as a bridge between contiguous platelets.

An analogous phenomenon may be involved in adhesion of platelets to artificial surfaces. The traditional view is that of Chiu et al,[14] who suggested that platelet interaction with surface-bound fibrinogen depends on the conformational alteration of the protein, induced by the energy of adsorption. Numerous studies[8,14,31] have shown that surfaces can induce conformational changes in adsorbed fibrinogen and other proteins. It has become widely accepted that surfaces that bind and denature fibrinogen most extensively are the ones most likely to induce platelet adhesion and activation. However, evidence presented here indicates that the reactivity of platelets with at least some artificial surfaces depends on the ability of the surfaces to adsorb a protein film containing fibrinogen with its functional domains maintained in a recognizable, minimally altered conformation.

For each of the three groups of polymers tested, platelet retention correlated positively with the amount of bound fibrinogen that remained recognizable by antifibrinogen antibodies. For the polyalkyl acrylates and for a group of unrelated polymers (PMMA, Biomer, and polystyrene) *total* fibrinogen binding also correlated with platelet retention. But for the polyalkyl methacrylates a positive correlation between platelet retention and antifibrinogen antibody binding obtained even though total fibrinogen binding kinetics were virtually indistinguishable for the four materials. Thus, the maintenance of fibrinogen's native conformation may be important in platelet-surface interactions, even though for some polymers *total* fibrinogen binding is also correlated with platelet retention. In addition, the fact that the hierarchy of platelet interaction with the polyalkyl methylacrylates was maintained even when platelet aggregation was blocked argues that this correlation is likely due to the direct mediation of platelet adhesion by "unaltered" fibrinogen on the surfaces.

The fibrinogen adsorption kinetics for methacrylate polymers exposed to whole or diluted plasma are similar to those reported by Vroman et al[12] for glass surfaces and by Brash et al[13] for glass, siliconized glass, and polyethylene. These kinetics indicate that some of the fibrinogen, bound after short incubation times, is removed from the surface, possibly via competition with other plasma proteins. Vroman et al[12] have presented evidence that high molecular weight kininogen is responsible for this effect. This apparent binding and removal of fibrinogen from the methacrylate polymers is also reflected in the measurements of antifibrinogen antibody binding.

The hierarchy of antifibrinogen antibody binding to the fibrinogen-coated methacrylate polymers was only observed when the fibrinogen-coated polymers were exposed to low concentrations of the antibody. As the antibody concentration was increased, antibody binding increased to ~0.25 $\mu g/cm^2$ (similar to the level of bound fibrinogen) and was indistinguishable for the four methacrylates. These results indicate that the differences in antibody binding on different polymers are likely due to differences in binding affinities of the antibody for the adsorbed fibrinogen molecules rather than to differences in the number of antibody-recognizable binding sites. Identical hierarchies of antibody binding to the fibrinogen-coated methacrylates were seen with antibodies against whole fibrinogen and also with anti-D region and anti-E region antibody preparations, suggesting that the different methacrylate polymers were not binding fibrinogen molecules in different orientations. Similar experiments using monoclonal antibodies may provide more definitive answers regarding the relative contributions of molecular orientation *v* conformational alteration to the antibody binding capacities of surface-bound fibrinogens. However, these observations could be explained if the different methacrylate polymers were inducing subtle, but distinguishable (by the antibodies) changes in the overall conformation of the bound fibrinogen molecules.

Chuang et al[32] examined two commonly used hemodialysis membranes, cuprophan and polyacrylonitrile, and found that platelet retention by these two surfaces was negatively correlated with total fibrinogen binding but positively correlated with the amount of surface-bound fibrinogen detected with antifibrinogen antibodies. Although Chuang and associates did not draw the same conclusions we have reached regarding the importance of fibrinogen's native conformation in the reactivity of the adsorbed protein with platelets, their data are compatible with this hypothesis.

If adsorbed fibrinogen does mediate platelet adhesion to surfaces, is prior activation of the platelet (perhaps by ADP) required or is the interaction of the platelet with adsorbed fibrinogen itself sufficient to induce activation? Red cell hemolysis or mechanical effects on platelets could produce low levels of ADP near the blood/artificial surface interface,[33] but in that case one must ask why platelet fibrinogen receptors exposed by this ADP would not already be occupied by soluble plasma fibrinogen. If platelet reactivity with artificial surfaces results from the interaction of nonactivated platelets with "native" adsorbed fibrinogen, how does such an interaction occur? For all of the polymers tested, total fibrinogen binding ranged between 0.2 $\mu g/cm^2$ and 0.5 $\mu g/cm^2$. Assuming dimensions of 60 A by 450 A for a rod-shaped fibrinogen molecule, close-packed monolayers would range from 0.2 $\mu g/cm^2$ for side-on attachment to 1.6 $\mu g/cm^2$ for end-on attachment.[34] At saturation the concentration of fibrinogen in this surface layer would approach 350 mg/mL. Such close-packing of fibrinogen, perhaps with a particular spatial distribution of "native" reactive sites, could explain the reactivity of these surfaces with platelets.[35] Such a bound array might behave as a multivalent ligand with the fixed fibrinogen molecules acting to crosslink fibrinogen receptors in the platelet membrane and inducing platelet activation in a manner similar to the "mobile receptor" hypothesis of Jacobs and Cuatrecasas.[36] Low fibrinogen binding affinities of nonactivated platelet binding sites would not preclude such a model. Hornick and Karush[37] have shown that anti-DNP (2,4-dinitro-phenyl) antibodies, with a monovalent association constant of 6×10^6 $(mol/L)^{-1}$, bind to DNP-coated bacteriophage with an association constant of 3.5×10^{11} $(mol/L)^{-1}$. Such effects of polyvalency have been treated theoretically by Crothers and Metzger[38] and similar mechanisms have been proposed by Santoro and Cunningham[39] to explain collagen-induced platelet aggregation and by Luscher et al[40] and Ganguly et al[41] to explain platelet activation by large molecules with repeating structures and by thrombin.

If surface-bound fibrinogen molecules do act as multivalent ligands to bind and activate platelets, then efforts to develop nonthrombogenic materials must strive to produce surfaces that, when in contact with plasma, will adsorb a protein film notable for its lack of fibrinogen maintained in a conformationally unaltered state.

ACKNOWLEDGMENT

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Platelet Aggregation by Fibrinogen Polymers Crosslinked Across the E Domain

By G. McManama, J.N. Lindon, M. Kloczewiak, M.A. Smith, J.A. Ware, J. Hawiger, E.W. Merrill, and E.W. Salzman

There is evidence that platelet interactions with artificial surfaces are mediated by plasma proteins, especially fibrinogen, adsorbed on the surfaces. Multiple site interactions between fibrinogen molecules adsorbed in high concentration and receptors in the unactivated platelet may be sufficient for platelet adhesion and subsequent activation. To examine this hypothesis, we prepared soluble polymers of fibrinogen. Polymers produced by interaction of fibrinogen with Fab'$_2$ fragments of antibodies against fibrinogen's E (central) domain (Fg-Fab'$_2$(E)) induced, in gel-filtered platelets, aggregation and serotonin release, which were blocked by monoclonal antibodies against the GPIIb/IIIa complex, by Fab fragments against the D domain, and by metabolic inhibitors; aggregation was attenuated but not abolished by enzymatic removal of ADP (with CP/CPK) or by blockage of ADP binding sites (with FSBA), and when secretion was inhibited by aspirin. Fg-Fab'$_2$(E) also induced a dose-dependent elevation in cytoplasmic Ca^{2+} (measured by Aequorin luminescence) which was attenuated by CP/CPK and by FSBA, and was eliminated by metabolic inhibitors and by anti-IIb/IIIa antibody. Fibrinogen complexes crosslinked with dimethylsuberimidate or Factor XIII neither aggregated gel-filtered platelets nor inhibited platelet aggregation by ADP and fibrinogen, probably because of inaccessability of lysine residues in the D (terminal) domain of fibrinogen, which are thought to be required for platelet binding. Thus, soluble complexes of fibrinogen having multiple available platelet receptor recognition sites activate gel-filtered platelets and may provide a useful model for platelet-surface interactions mediated by adsorbed fibrinogen.

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WHEN an artificial surface is exposed to blood, it rapidly acquires an adsorbed film of plasma proteins, which precedes the interaction of the surface with blood cells, including platelets.[1-3] Considerable evidence supports the hypothesis that adsorption of a specific protein, fibrinogen (Fg), by artificial surfaces is a prerequisite for subsequent platelet adhesion.[4-10] Brash et al[11,12] observed that fibrinogen is often preferentially adsorbed from mixtures with albumin or gammaglobulins and that the fibrinogen-to-albumin ratio in the adsorbed layer correlates with platelet reactivity induced by many surfaces. Coller[13] reported that latex particles coated with fibrinogen agglutinate gel-filtered platelets (GFP) without the addition of other platelet agonists. In our laboratory in vitro studies of platelet retention on bead columns have also indicated that although albumin precoating of the beads reduces platelet reactivity (retention), fibrinogen precoating markedly augments it.[14]

On the other hand, platelets are normally bathed in a fibrinogen solution in the blood but do not bind appreciable quantities of fibrinogen in blood in vitro, and presumably not in the circulation in vivo, unless they are stimulated by agonists such as adenosine diphosphate (ADP), thrombin, or epinephrine.[15-19] The receptor for fibrinogen on stimulated platelets has been identified as the glycoprotein IIb/IIIa complex[20-24]; Hawiger et al[25-28] and Plow and associates[29,30] have suggested that a locus on the carboxy-terminal region of the fibrinogen gamma chain is the primary binding site for this receptor complex. The possibility that fibrinogen may interact with two classes of platelet receptors, those with high or low affinity, has been suggested.[17,31,32]

Why platelets require activation before reacting with fibrinogen in fluid blood but apparently not with fibrinogen adsorbed on a surface has been puzzling. A popular explanation is that platelet interactions with surface-adsorbed fibrinogen result from an alteration in the conformation of the fibrinogen molecule as a result of its adsorption on the surface. Conformational changes in fibrinogen adsorbed on various surfaces have in fact been described.[33-36] However, Lindon et al[37] have shown that platelet interaction with fibrinogen-coated polyalkyl methacrylate surfaces correlated with the concentration of antibody-detectable or "native" fibrinogen on the surface rather than with the total amount of fibrinogen bound, suggesting that preservation of recognizable platelet-binding loci on adsorbed fibrinogen molecules may be important in platelet adhesion. Similarly, Chuang et al[38] have suggested that variable retention of the conformational integrity of adsorbed fibrinogen may explain differences in platelet adhesion to surfaces of dialysis devices.

It is accepted that fibrinogen acts as a cofactor in platelet aggregate formation after the activation of the platelets by ADP or other agonists has resulted in exposure of the glycoprotein IIb/IIIa receptors. However, it is also possible that fibrinogen molecules arrayed on a surface may themselves activate platelets and facilitate platelet adhesion. When adsorbed on a surface, proteins may be present in greatly increased local concentration: eg, a surface that has adsorbed 1 μg Fg/cm^2 has a surface fibrinogen concentration equivalent to that of a solution containing 220 mg/mL.[39] Furthermore, closely arrayed molecules adsorbed on a surface may simultaneously present a multitude of specific binding sites for cell membrane receptors. Although individual sites may interact only weakly with "low affinity" receptors, this "multivalency" of available sites may result in binding which in the aggregate is quite strong.[40] Finally,

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adsorption on a surface may induce conformational changes in a protein that may either enhance or reduce the accessibility or recognition of a binding site.[33-36]

As a way of testing the possible effects that high local concentrations of fibrinogen with multiple platelet-binding loci might have upon platelets, we sought to prepare soluble polymers of fibrinogen that had these characteristics. These polymers, not to be confused with fibrin, might be considered as models of fibrinogen adsorbed on an artificial surface. Although these soluble polymers might not be identical in conformation to fibrinogen that has become surface-bound, the multivalent presentation of platelet binding loci may mimic events that take place when circulating platelets interact with protein-coated surfaces.

MATERIALS AND METHODS

Materials. Human fibrinogen and plasmin were obtained from Kabi, Helena Laboratories (Beaumont, Tex). Human fibrinogen was also obtained from US Biochemicals (Cleveland, Ohio) as were goat Fab'_2 fragments specific for rabbit IgG heavy and light chains. Sea-gamma-alumina gel, L-phenylalanyl-L-prolyl-L-arginyl-chloromethylketone (PPACK), and Freund's adjuvant were purchased from Calbiochem (La Jolla, Calif). Epsilon amino caproic acid, benzamidine hydrochloride, triethanolamine, HEPES, bovine serum albumin, aprotinin, apyrase, phosphocreatine, creatine phosphokinase, 5'-fluorosulfonylbenzoyladenosine (FSBA), fetuin-agarose, lysine-agarose, Protein A-Sepharose, and 2-deoxyglucose were obtained from Sigma (St. Louis, Mo). DEAE-Sephacel, CM-Sepharose, Sepharose 2B and 4B, Sephadex G25 and G75, and Sephacryl S-200 and S-500 were purchased from Pharmacia (Piscataway, NJ). From Pierce Chemical (Rockville, Ill), dimethylsuberimidate and Iodogen were obtained. Parke-Davis (Morris Plains, NJ) was the supplier for human thrombin. Worthington (Freehold, NJ) provided papain and pepsin. The molecular weight standardization kits for SDS-PAGE were obtained from Biorad (Rockville Center, NY) and Bethesda Research Laboratories (Gaithersburg, Md). Aequorin was obtained from Dr John Blinks, Mayo Clinic, Rochester, Minn. Murine monoclonal antibodies in ascites fluid were generously donated by Dr Barry Coller (Dept of Hematology, State University of New York at Stony Brook, NY). Outdated citrated plasma was a gift of the Beth Israel Hospital blood bank.

Preparation of human fibrinogen. Human fibrinogen was prepared from pooled outdated citrated plasma or from partially purified commercial products by a modification of the method reported by Wang.[41] Citrated human plasma was stirred slowly (4 °C) with a slurry of alumina gel sufficient to increase the prothrombin time to greater than 70 sec (~500 mg gel/100 mL plasma). After centrifugation (20,000 g, 30 min, 4 °C) the supernatant was decanted. When commercial human fibrinogen was employed, it was initially dissolved in buffer BEC (55 mmol/L sodium citrate, 100 mmol/L epsilon-amino caproic acid, and 10 mmol/L benzamidine HCl). The subsequent purification scheme was similar for commercial and plasma sources of fibrinogen. Precipitation with lysine (to 0.2 mol/L) and glycine (to 2.0 mol/L) was performed with slow stirring (4 °C, 3 hr) after which the precipitate was recovered by centrifugation (20,000 g, 30 min, 4 °C). The precipitation was repeated after dissolving the pellet in BEC (~8 mg/mL). After recovery the precipitate was dissolved in PCBS (21 mmol/L sodium phosphate, pH 6.5, 72 mmol/L sodium chloride, 0.5 mmol/L KCN), dialyzed (4 °C, 24 hr), and centrifuged (25,000 g, 1 hr, 4 °C), and the supernatant was passed over lysine-agarose and eluted with PBS (0.1 mol/L sodium phosphate, pH 7.4). The fibrinogen solution was concentrated by lysine-glycine precipitation, centrifugation, and redissolution in 0.3 mol/L NaCl (15 to 20 mg/mL). Concentration was measured by spectrophotometric absorbance at 280 nm using an extinction coefficient of 15.3. After exhaustive dialysis against 0.3 mol/L NaCl, the solution was stored in aliquots at −80 °C. Clottability was >96%. Examination by reduced SDS-PAGE (method of Laemmli[42]) revealed a doublet A-alpha band of M_r 68,000, singlet B-beta band of M_r 54,000, and singlet gamma band of M_r 47,000. No significant contaminants were seen: ie, <1% Factor VIII:vWF, fibronectin, and fibrin.

Preparation of fibrinogen D and E fragments. Plasmin digestion of fibrinogen was performed in the presence of calcium chloride (50 mmol/L) and PPACK (1 μmol/L) according to previously described procedures.[23,43] Purity of fragments was verified by nonreduced SDS-PAGE on linear gradient gels (3% to 15%) which revealed a single band (M_r 94,000) for the D fragment and a single band (M_r 48,000) for the E fragment.

Preparation of antihuman fibrinogen antibodies. Healthy male rabbits were injected intramuscularly with human fibrinogen (500 μg) in Freund's adjuvant (500 μL), followed by a second injection 4 weeks later. Booster immunizations were given intramuscularly at 4-week intervals. Three to seven days following booster administration the rabbits were bled from the central artery of the ear. The serum IgG fraction was removed by ammonium sulfate precipitation (42% saturation) and then purified by affinity chromatography using columns of Fg-Sepharose, fragment-D-Sepharose, and fragment-E-Sepharose, prepared according to the procedure of Cuatrecasas.[44] Antibodies were separated into fractions recognizing fibrinogen and E fragment (but not D), and fibrinogen and D fragment (but not E). Cross-reactivity of antibodies for D fragment and E fragment was assessed by Western gel blotting techniques[45,46] and was not detectable. The fragment-specific antibodies were dialyzed against distilled water, lyophilized, and stored at −80 °C.

Preparation of fibrinogen polymers. Preparation of Fg-DMS polymer by crosslinking with dimethylsuberimidate (DMS). DMS was dissolved (100 mmol/L) in triethanolamine (0.5 mol/L) at pH 8.5. Fibrinogen was diluted to 5 mg/mL in 10 mL of triethanolamine (0.5 mol/L, pH 8.5). At 37 °C, DMS was added in 500 μL portions to the fibrinogen solution and rapidly mixed. After five minutes, 4 mL of 500 mmol/L ammonium acetate was added to quench the unreacted DMS, the mixture was centrifuged at 20,000 g for 15 minutes, and the supernatant was rapidly exchanged into 0.3 mol/L NaCl by passage through a column of G25 Sephadex. The products of reaction were electrophoresed under nonreducing conditions with sodium dodecyl sulfate (SDS) on 3% polyacrylamide slab gels according to the method of Laemmli[42] and stained with Coomassie blue. Estimation of relative molecular weights based upon relative band mobilities indicated that a distribution of covalently associated fibrinogen oligomers had been formed, with the largest distinguishable species being pentameric. Dimeric and trimeric oligomers predominated. Clottability of the unfractionated population of Fg-DMS oligomers was 60%, a marked decrease from that of the fibrinogen monomer precursor.

Preparation of fibrinogen cross-linked by activated factor XIII (Fg-F13). Factor XIII was purified from human plasma according to the procedure of Kazama et al[47] and employed to crosslink fibrinogen monomers by the method of Kanaide et al.[48] Calcium chloride (3.2 mmol/L) and human thrombin (10 U/mL) were added to Factor XIII (0.8 mg/mL) in 0.1 mol/L sodium phosphate, pH 7.4 (PBS), and incubated (37 °C, 20 min). PPACK (10 μmol/L) was then added, vigorously mixed, and incubated (37 °C, 10 min), after which the mixture was centrifuged (10,000 g, 15 min, 20 °). To the supernatant, calcium chloride (15 μmol/L) and purified fibrinogen (6 mg/mL) were added; the mixture was incubated (37 °, 60 min), and EDTA added (8 mmol/L). After rapid exchange into PBS using G25 Sephadex, the unfractionated mixture was stored at −80 °C. Clottability of the unfractionated mixture was approximately 85%. Analysis on nonreduced 3% SDS-PAGE revealed a distribution of fibrinogen polymeric species with predominantly dimers and trimers and detectable amounts of tetramers and pentamers. Clottability of this population of Fg-F13 oligomers was 85%.

Preparation of Fg-Fab$'_2$(E) (two components) and Fg-Fab(E)-Fab$'_2$ (three components) fibrinogen polymers by crosslinking with antifibrinogen antibody fragments. Preparation of fibrinogen polymers with E-domain–specific antibodies was undertaken out of concern that alteration of native fibrinogen loci, necessary for binding to platelet receptors for fibrinogen, might occur during nonspecific chemical or enzymatic crosslinking. Monovalent (Fab) and divalent (Fab$'_2$) antibody fragments were prepared using procedures of Porter[49] and Nisonoff[50] as previously described.[43] Fg-Fab$'_2$ (E) polymer was prepared by mixing E-fragment–specific Fab$'_2$ and fibrinogen in a 2:1 molar ratio, which resulted in less than 2% precipitation at 22 °C. Fg-Fab(E)-Fab$'_2$ polymer was prepared by mixing antifibrinogen Fab (produced in rabbit), goat Fab$'_2$ (against rabbit Fab), and fibrinogen in a 2:3:1 molar ratio. Less than 10% of the Fg-Fab(E)-Fab$'_2$ polymer precipitated at 22 °C.

Passage of the Fg-Fab$'_2$(E) polymer over a Sepharose 4B size exclusion chromatography column produced a single peak at the void volume, indicating that the apparent molecular size of the complex was equal to or greater than that of a globular protein with M_r 20 × 10^6 or a polysaccharide chain of M_r 5 × 10^6. Similar experiments with the Fg-Fab(E)-Fab$'_2$ polymer resulted in dissociation of the complexes during chromatography. Attempts to estimate the macromolecular size of the Fg-Fab(E)-Fab$'_2$ and Fg-Fab$'_2$(E) polymers by nonreduced SDS-PAGE on 3% gels resulted in dissociation of the polymer. Nondenaturing electrophoresis on hybrid 0.5% agarose/1.5%–3% acrylamide gels revealed failure of the fibrinogen polymers to penetrate even these large pore gels.

Platelet preparation. Blood was obtained by atraumatic venipuncture from healthy volunteer donors who denied having taken aspirin or other drugs affecting platelet function during the preceding 2 weeks and was anticoagulated with 3.8% sodium citrate (9:1). After centrifugation (2,000 g, 2 min) prostaglandin E_1 (PGE_1) was added to 20 mL of platelet-rich plasma (PRP) and the platelets were pelleted by centrifugation (425 g, 15 min, 20 °). The pellet was resuspended in 1 mL HEPES-Tyrodes buffer (129 mmol/L sodium chloride, 8.9 mmol/L sodium bicarbonate, 2.8 mmol/L potassium chloride, 0.8 mmol/L potassium phosphate, 0.8 mmol/L magnesium chloride, 2.4 mmol/L calcium chloride, 10.9 mmol/L sodium citrate, 5.6 mmol/L glucose, 10 mmol/L HEPES, and 0.35% bovine serum albumin), to which PGE_1 had been added. The resuspended platelets were then washed by passage over a 12 mL Sepharose 2B column preequilibrated with HEPES-Tyrodes, and the gel-filtered platelets (GFP) were diluted with HEPES-Tyrodes to a count of 200,000/μL. Aequorin-loaded platelets (ALP) were prepared as described by Johnson et al[51] and measurements of ionized cytoplasmic Ca^{2+} by aequorin luminescence were conducted as described.[52,53]

Aggregometry. A Sienco model DP-247E dual channel aggregometer was employed for studies of platelet aggregation,[54] and the presence of platelet aggregates was confirmed by phase microscopy. The production of platelet microaggregates observed in experiments with the Fg-Fab(E)-Fab$'_2$ polymer was quantified by measuring the number of residual platelet "singlets" in GFP after addition of the polymer (300 μg/mL), after the method of Kohanna et al.[55] Secretion of ^{14}C-serotonin was measured by a published method.[56]

Inhibitor studies. The ADP-scavenging system employing creatine phosphokinase (20 U/mL) and creatine phosphate (10 mmol/L) to convert ADP to ATP[57] was used to investigate the role of ADP in the generation of platelet responses to fibrinogen polymer. Apyrase (purified by passage over fetuin-agarose[58]) was also employed in an empirically determined amount sufficient to inhibit ADP-induced platelet aggregation in GFP (5 μmol/L ADP, 500 μg/mL Fg). 5′-fluorosulfonylbenzoyl adenosine (FSBA), a covalent inhibitor of ADP that inhibits binding of ADP to platelets,[59,60] was dissolved in methanol, added to GFP (final concentration 100 μmol/L), and allowed to incubate for five minutes before addition of agonists. Sodium azide (10 mmol/L) and 2-deoxyglucose (10 mmol/L) were employed as inhibitors of platelet energy metabolism.[61,62] Dissolved in saline, these were added to unstirred GFP at 22 °C and incubated for at least 30 minutes before use. Control GFP, incubated for 30 minutes at 20 °C without the inhibitors, remained normally responsive to agonists. The murine monoclonal antibody 10E5, which binds specifically to the platelet membrane glycoproteins IIb/IIIa,[63] was purified from ascites fluid by affinity chromatography on Protein-A Sepharose as described by Ey et al.[64] In some experiments aspirin was added to GFP and incubated for 30 minutes at 22 °C.

RESULTS

Aggregation of platelets. The effect of the polymer preparations on stirred suspensions of gel-filtered platelets was tested at 37 ° in an aggregometer. Control samples of GFP to which 500 μg/mL fibrinogen followed by 5 μmol/L ADP were added, aggregated normally (Figs 1 and 2). Addition of fibrinogen alone (Fig 2) induced no change in light transmission, suggesting that the platelets were not already significantly activated (eg, by ADP liberated during gel filtration). Addition of ADP (5 μmol/L) alone induced shape change, as indicated by a fall in light transmission, but did not produce platelet aggregation, suggesting that the GFP did not contain significant residual fibrinogen in the medium.

Addition of Fg-DMS (50 μg/mL) to GFP did not produce platelet aggregation or shape change, nor did it substitute for

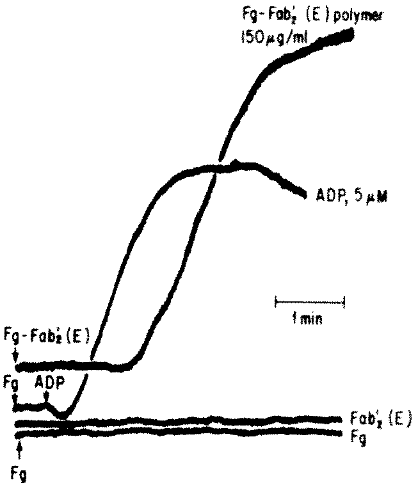

Fig 1. Aggregation of gel-filtered platelets by Fg-Fab$'_2$(E) polymer and lack of aggregation by its components. Aggregation in response to Fg-Fab$'_2$(E) polymer (150 μg/mL) or fibrinogen (500 μg/mL) and ADP (5 μmol/L) is contrasted to the lack of response to Fab$'_2$(E) antibody (300 μg/mL) alone or fibrinogen (500 μg/mL) alone.

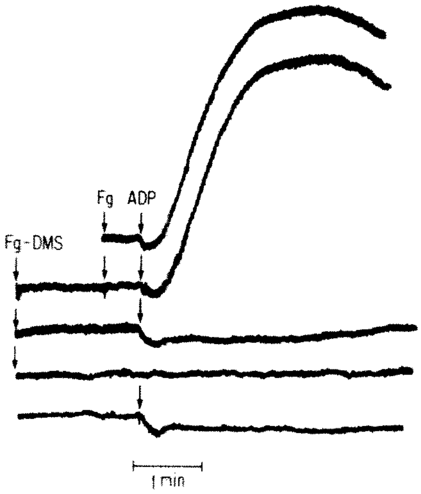

Fig 2. Effects of Fg-DMS polymer on the aggregation of gel-filtered platelets. Lack of aggregation in response to Fg-DMS (50 µg/mL) alone or Fg-DMS (50 µg/mL) followed by ADP (5 µmol/L) is contrasted with aggregation in response to fibrinogen (500 µg/mL) and ADP (5 µmol/L), or to Fg-DMS (50 µg/mL) followed by fibrinogen (500 µg/mL) and ADP (5 µmol/L).

fibrinogen in support of platelet aggregation after addition of ADP (5 µmol/L) to the stirred mixture (Fig 2). Addition of Fg-DMS, then fibrinogen, and then ADP (5 µmol/L) to GFP did not reveal any inhibition of aggregation by the Fg-DMS polymer, and was not different from aggregation of platelets by ADP (5 µmol/L) in the presence of fibrinogen (500 µg/mL). Results obtained with Fg-F13 (20 µg/mL) were essentially the same as for Fg-DMS (data not shown). Thus, neither Fg-DMS nor Fg-F13 stimulated platelet aggregation, and neither supported or inhibited ADP-induced aggregation.

In contrast, addition of Fg-Fab$'_2$(E) over a range of 35 µg/mL to 280 µg/mL aggregated GFP in a dose-dependent fashion (Fig 1, 3). Addition of exogenous ADP was not required for this response. Addition of anti-E–fragment Fab$'_2$ alone at concentrations up to 300 µg/mL did not produce platelet aggregation. Additions of fibrinogen alone at concentrations up to 500 µg/mL also produced no detectable aggregation. Addition of Fg-Fab$'_2$(E) (140 µg/mL) to citrated platelet-rich plasma also caused platelet aggregation (data not shown).

Platelet aggregation in response to the three component (Fg-Fab(E)-Fab$'_2$) polymer was also studied. When Fg-Fab(E)-Fab$'_2$ polymer was added to stirred GFP at 37 °C there was a small increase in light transmission (data not shown). Phase microscopic examination of samples from the aggregometer cuvette showed many small platelet aggregates (estimated to contain 3 to 10 platelets) in samples containing Fg-Fab(E)-Fab$'_2$, but not in the controls. These aggregates were quantified by measuring the number of residual "singlet" platelets by the method of Kohanna et al.[55] In this method, doublets are counted as single platelets. After exposure to Fg-Fab(E)-Fab$'_2$, 70% of platelets were measured in the "singlet" window, compared with 96% of those exposed to fibrinogen alone and 100% of those exposed to the Fab against fragment E alone.

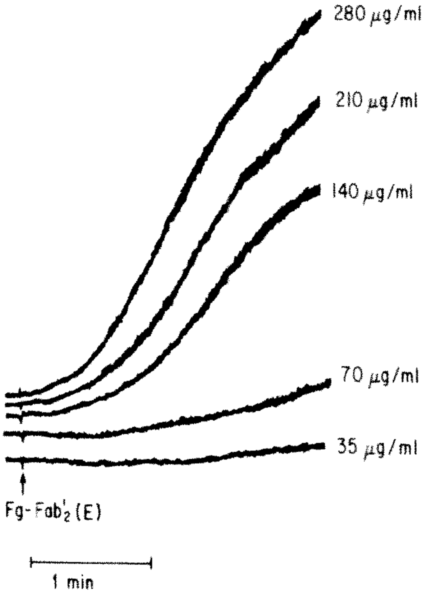

Fig 3. Dose response of Fg-Fab$'_2$(E) polymer-induced aggregation of gel-filtered platelets. Values indicated are final concentrations.

These experiments indicated that, although neither monomeric fibrinogen nor the fragment-E–specific antibody fragments could induce platelet aggregation, a polymer produced by combination of the two in an appropriate ratio was sufficient stimulus to produce platelet aggregation. When fibrinogen was added to GFP followed by addition of Fab$'_2$(E), in final concentration equivalent to those present during addition of Fg-Fab$'_2$(E) polymer, the same extent of aggregation of GFP was also seen, following a two- to three-minute lag period (Fig 4). The same result was found when addition of fibrinogen was preceded by Fab$'_2$(E), but

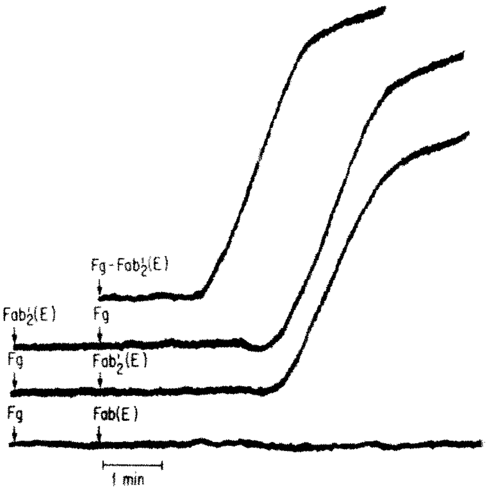

Fig 4. Aggregation of gel-filtered platelets by the components of Fg-Fab$'_2$(E) polymer added separately. Aggregation in response to Fg-Fab$'_2$(E) polymer (140 µg/mL), fibrinogen (90 µg/mL) followed by Fab$'_2$ (50 µg/mL), Fab$'_2$ (50 µg/mL) followed by fibrinogen (90 µg/mL), or fibrinogen (90 µg/mL) followed by Fab(E) (50 µg/mL) are shown for comparison.

not with fibrinogen and monovalent Fab specific for the E fragment. Neither fibrinogen nor Fab$'_2$(E) alone aggregated platelets. These results suggest that interaction of fibrinogen with Fab$'_2$(E) (ie., polymer formation) was necessary for platelet aggregation in these experiments.

Effect of inhibitors of platelet aggregation. To explore the possibility that the clumping of platelets observed after addition of fibrinogen polymer was due to passive platelet agglutination, GFP were incubated for 30 minutes at 20 °C with metabolic inhibitors sodium azide (10 mmol/L) and 2-deoxyglucose (10 mmol/L), which have been shown to inhibit platelet metabolic processes.[61,62] Under these conditions GFP failed to aggregate after addition of Fg-Fab$'_2$(E) polymer (35–280 μg/mL) (Fig 5). They also showed no shape change or aggregation after addition of fibrinogen (500 μg/mL) and ADP (5 μmol/L). A simultaneous control sample of GFP (no inhibitor, incubated 30 minutes at 20 °C) aggregated similarly to fresh GFP. These results suggested that aggregation of platelets by the Fg-Fab$'_2$(E) polymer was an active, energy-requiring process that could not be attributed to agglutination alone.

We examined the possible role of ADP in the stimulation of platelets by the Fg-Fab$'_2$(E) polymer. The covalent inhibitor of ADP, FSBA (100 μmol/L), in methanol was added to stirred GFP and incubated at 37 °C for five minutes. Addition of fibrinogen (500 μg/mL) followed by ADP (10 μmol/L) to FSBA-treated GFP produced no aggregation. Fg-Fab$'_2$(E)–induced aggregation was inhibited in this system (Fig 5). Methanol was without effect at the concentration employed. In other experiments the ADP scavenging system, phosphocreatine (CP, 2 mmol/L) and phosphocreatine kinase (CPK, 10 U/mL), was added to GFP stirred at 37 °C. Addition of fibrinogen (500 μg/mL) and ADP (10 μmol/L) one minute after addition of CP/CPK produced only a small increase in light transmission, indicating virtually complete inhibition of aggregation. Addition of Fg-Fab$'_2$(E) polymer to GFP in the presence of CP/CPK resulted in attenuation of aggregation. Thus, it would appear that, although the Fg-Fab$'_2$(E) polymer can produce full-scale aggregation of GFP without addition of exogenous ADP, endogenous ADP plays a role in the aggregation response to the polymer.

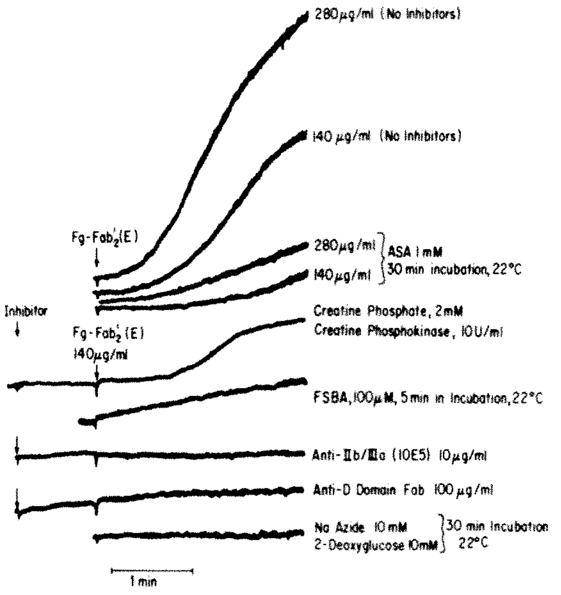

Fig 5. Effects of various inhibitors on Fg-Fab$'_2$(E) polymer-induced aggregation of gel-filtered platelets. For details, see text.

Monovalent Fab fragments specific for the D domain of fibrinogen have previously been shown to inhibit fibrinogen binding to ADP-stimulated platelets and to inhibit aggregation of GFP in the presence of fibrinogen and ADP.[65] We confirmed that anti-D–fragment Fabs inhibited aggregation of GFP by ADP (5 μmol/L) in the presence of fibrinogen (500 μg/mL) (anti-E–fragment Fabs were without effect; data not shown). When added to stirred GFP at 37 ° these anti-D–fragment Fabs (100 μg/mL) completely inhibited aggregation by the Fg-Fab$'_2$(E) polymer (Fig 5). This result indicated that aggregation of GFP induced by the Fg-Fab$'_2$(E) polymer required accessibility of the D domain of fibrinogen, either on the polymer itself or on fibrinogen which might be released by the platelet in response to stimulation by the polymer.

Since the previous experiments suggested that interaction of the Fg-Fab$'_2$(E) polymer with platelets involved the D domain of fibrinogen and therefore possibly fibrinogen's conventional platelet binding site (identified as residing in the D domain at the carboxy-terminal end of the gamma chain[28]), we studied the role of the platelet membrane receptor for fibrinogen, glycoprotein IIb/IIIa complex, in the aggregation of GFP by Fg-Fab$'_2$(E) polymer. The murine monoclonal antibody 10E5 was added (10 μg/mL) to platelets stirred at 37 °C in the aggregometer (Fig 5). After a one minute incubation, addition of fibrinogen (500 μg/mL) and ADP (5 μmol/L) produced no aggregation. At the same concentration, the 10E5 antibody also prevented aggregation of GFP by Fg-Fab$'_2$(E) polymer. This implies that the platelet membrane glycoprotein IIb/IIIa complex, target of the monoclonal antibody and presumed platelet fibrinogen receptor, is involved in the aggregation of GFP by the Fg-Fab$'_2$(E) polymer.

Gel-filtered platelets that had been incubated with aspirin for 30 minutes at 20 °C were also studied. The GFP failed to aggregate in response to arachidonic acid, and in response to fibrinogen (500 μg/mL) and ADP (5 μmol/L) they underwent primary aggregation but not secondary aggregation, as would be expected. Incubation of GFP with aspirin partially reduced the rate and extent of aggregation induced by the Fg-Fab$'_2$(E) polymer (Fig 5).

Release of serotonin. The effect of Fg-Fab$'_2$(E) polymer upon platelet secretion was tested with ^{14}C-serotonin–loaded GFP in an aggregometer. As shown in Table 1, serotonin release induced by Fg-Fab$'_2$(E) polymer was dose-dependent and paralleled the extent of aggregation. In contrast, addition of either fibrinogen or Fab$'_2$ specific for the E fragment produced little release of serotonin. Metabolic inhibition with sodium azide and 2-deoxyglucose blocked release of serotonin in response to Fg-Fab$'_2$(E). Inhibition of ADP with FSBA or CP/CPK produced substantial inhibition of the secretory response to Fg-Fab$'_2$(E). Blockade of the IIb/IIIa

Table 1. ^{14}C-Serotonin Release From GFP After Stimulation by Fg-Fab$'_2$(E)

Addition	Release (%)
Background (no addition)	3.0
Fibrinogen, 500 μg/mL	3.5
Fab$'_2$(E), 200 μg/mL	4.1
Fg-Fab$'_2$(E) polymer	
35 μg/mL	6.6
70 μg/mL	14.9
140 μg/mL	26.3
280 μg/mL	51.0
Fg-Fab$'_2$(E), 140 μg/mL after	
CP/CPK 10 mmol/L, 10 U/mL	6.5
FSBA, 100 μmol/L	5.0
Anti-IIb/IIIa (10E5)	3.2
Fab (D), 100 μg/mL	5.7
NaN_3/2-deoxy glucose, 10 mmol/L/10 mmol/L	8.5
PGE_1, 1 μmol/L	3.3
Aspirin, 1 mmol/L	9.8

Values represent percentage of ^{14}C-serotonin secreted by platelets, relative to total content in control platelets.

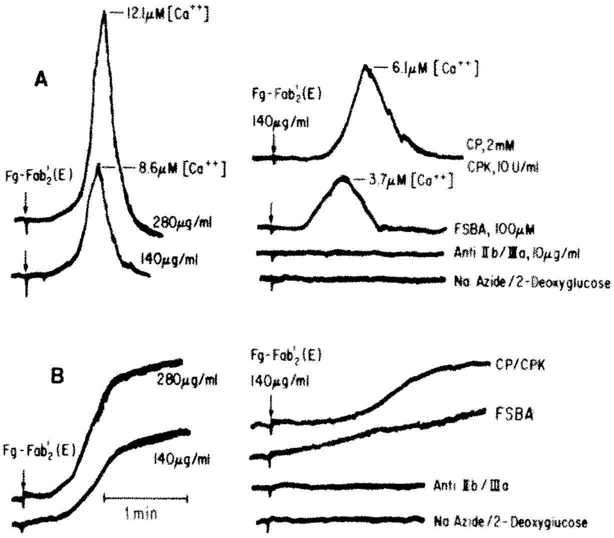

Fig 6. Effects of various inhibitors on Fg-Fab$'_2$(E) polymer-induced elevation of cytoplasmic calcium ion concentration. Calcium levels, measured by the aequorin luminescence technique (A) and platelet aggregation (B), were measured simultaneously. Peak calcium ion concentrations, determined as described in Materials and Methods, are indicated.

glycoprotein with the monoclonal antibody 10E5 prevented serotonin release, as did blockade of the D domain of the polymer with monovalent Fab specific for the D fragment. Aspirin pretreatment reduced the release of serotonin stimulated by Fg-Fab$'_2$(E). These observations supported the conclusion that GFP exposed to Fg-Fab$'_2$(E) polymer had not only become aggregated but that a transmembrane signal had been generated, producing internal activation that culminated in the release reaction.

Measurement of cytoplasmic free calcium concentration. With evidence that GFP exposed to the multivalent Fg-Fab$'_2$(E) polymer had become activated internally, it was of interest to determine whether the platelet cytoplasmic calcium ion concentration was elevated in response to the polymer, as is the case with platelet agonists such as thrombin and ADP.[53] Aequorin-loaded GFP were prepared[51] and agonists were added to suspensions in a modified Lumi-aggregometer (Chronolog, Inc, Boulder, Co). Simultaneous recordings of aequorin luminescence and optical density, representing platelet aggregation, were obtained.[52] As is seen in Fig 6, addition of the Fg-Fab$'_2$(E) polymer resulted in dose-dependent elevation of ionized calcium in the absence of exogenous ADP. Aggregation of aequorin-loaded platelets (ALP) followed the increase in calcium concentration. The rise in Ca^{2+} after addition of Fg-Fab$'_2$(E) was abolished when the polymer was added in the presence of the monoclonal anti-IIb/IIIa antibody 10E5 (10 μg/mL). Addition of CP/CPK (sufficient to block aggregation of ALP by 5 μmol/L ADP in the presence of fibrinogen (500 μg/mL)) strongly attenuated the extent of aggregation but only slightly reduced the increase in [Ca^{2+}]. Addition of FSBA, an inhibitor of ADP at the receptor level, inhibited aggregation and significantly reduced Ca^{2+} elevation. When Fg-Fab$'_2$(E) was added to ALP that had been metabolically inhibited by incubation with Na azide (10 mmol/L) and 2-deoxyglucose (10 mmol/L), no increase in cytoplasmic [Ca^{2+}] was observed. These data indicate that platelet cytoplasmic [Ca^{2+}] is increased by Fg-Fab$'_2$(E) by an energy-dependent mechanism, that this increase is a consequence of Fg-Fab$'_2$(E) polymer interaction with the GP IIb/IIIa receptor, and that release of endogenous ADP enhances the response but is apparently not required for the rise in [Ca^{2+}] to occur.

DISCUSSION

In these experiments we have prepared polymers of fibrinogen by several crosslinking techniques. Polymers prepared by methods that may alter the D-domain binding site failed to induce aggregation of GFP and failed to support GFP aggregation after stimulation with ADP. Polymers prepared by crosslinkage with DMS or activated Factor XIII did not support platelet aggregation after stimulation with ADP, probably because DMS reacts with lysine residues that are integral to the recognizability of the gamma chain locus for receptor binding,[25-28] and because Factor XIII crosslinkage also affects this region.[66] In contrast a polymer, Fg-Fab$'_2$(E), prepared by a method that crosslinked fibrinogen domains thought to be unnecessary for platelet binding, was a sufficient and potent stimulus to aggregation of GFP. A related experiment was reported by Tollefson and Majerus[67] who found that divalent anti-fibrinogen antibodies promoted secretion of platelets on which fibrinogen was adsorbed, but that monovalent antibody fragments to fibrinogen did not induce platelet activation. The cross-linkage of fibrinogen receptor complexes by divalent antibody could have produced platelet activation and aggregation similar to that seen in our results.

In our experiments platelet aggregation was blocked by a monovalent antibody fragment specific for the fibrinogen D fragment. These observations are consistent with localization of the primary fibrinogen binding site for platelets in the D region. The further observation of blockade of fibrinogen polymer-induced aggregation by the monoclonal antibody 10E5 (which has previously been shown to block fibrinogen binding to the ADP-induced platelet receptors for fibrinogen[63]) suggests that the polymer may function by binding to the IIb/IIIa receptor and perhaps cause platelet activation by receptor crosslinkage or other mechanism. The aggregation by Fg-Fab$'_2$(E) was dose dependent and irreversible. The effect was attenuated by metabolic inhibitors, variably reduced by removal of ADP or blocking of ADP binding sites and partially inhibited by aspirin pretreatment. Initiation of platelet aggregation by the polymer was associated with a marked rise in cytoplasmic calcium ion concentration, as measured by aequorin luminescence. Release of serotonin was induced by the polymer, and variable degrees of inhibition of serotonin release were seen after metabolic inhibition, blockade or enzymatic digestion of ADP, blockade of fibrinogen receptors with monoclonal antibody, and inhibition of cyclooxygenase activity with aspirin.

That the aggregation of GFP by Fg-Fab$'_2$(E) was attenuated by ADP scavenging systems or after incubation with FSBA suggests that ADP release from the platelet may mediate or facilitate the aggregation response to the polymer. The observation that no aggregation, serotonin release, or elevation of cytoplasmic free calcium ion was seen in response to fibrinogen alone is evidence against the possibility that these GFP were already subjected to ADP stimulation before exposure to the Fg-Fab$'_2$(E) polymer. Thus the polymer itself appears to be capable of stimulating the platelet initially, presumably by binding to fibrinogen receptors, without dependence on the receptor-inducing effects of either exogenous or platelet-derived ADP.

The increase in affinity of the platelet for fibrinogen after addition of ADP is thought to involve exposure of the platelet fibrinogen binding sites, perhaps via a change in conformation of the GP IIb/IIIa complex. Newman et al[68] have shown that glycoproteins IIb and IIIa are associated in a 1:1 stoichiometry in the plasma membrane of intact, nonactivated platelets. Coller[69] has recently presented evidence that "unmasking" of the fibrinogen receptor binding site is the result of changes in the conformation or microenvironment of the IIb/IIIa complex. He observed that rates of binding of small antibody fragments (Fab$'_2$ and Fab), prepared from a monoclonal antibody (7E3) specific for the IIb/IIIa receptor of unactivated platelets, were increased in proportion to their smaller size. These findings may be indications that fibrinogen is weakly associated with the resting GP IIb/IIIa receptor complex. However, according to our model, presentation of multiple fibrinogen reaction sites at high local concentration in native conformation may have sufficient multivalent affinity to lead to binding and activation despite the relative inaccessibility of the receptor binding site for fibrinogen in the resting platelet.

Polymers composed of repeating subunits, each of which expresses binding sites for cell membrane receptors, may behave quite differently in biological systems from the same number of unassociated subunits acting independently. The effects of "multivalency" of binding loci upon receptor affinity have been analyzed by Crothers and Metzger,[40] who concluded that, as a general principle, multivalent ligands may have affinities for their receptors that are several orders of magnitude higher than corresponding monovalent ligands. This prediction has been verified experimentally by Hornick and Karush,[70] who determined that divalent anti-DNP (2,4-dinitrophenol) antibodies have K_D of 2.9×10^{-12} mol/L compared with 1.67×10^{-7} mol/L for the monovalent Fab fragment.

The mobile receptor hypothesis of Jacobs and Cuatrecasas[71] states that membrane receptors may be freely diffusible laterally in the resting state but when bound by ligands may become associated into complexes that promote effector activities. Receptor concentration ("patching") stimulated by binding of multivalent ligands has been observed in several cell systems.[72,73] Steric rearrangement of receptors has also been offered as an explanation for platelet behavior.[74-76] Polley[77] published elegant electron micrographs documenting the formation of clusters of GP IIb/IIIa complexes in response to platelet stimulation by thrombin. DeMarco et al[78] recently suggested that platelet interaction with type IIb von Willebrand factor may result in clustering of the IIb/IIIa receptor complex and facilitate platelet-fibrinogen binding and platelet aggregation. These observations are notably analogous to those we describe with the Fg-Fab$'_2$(E) polymer.

Although it is possible that platelet activation by fibrinogen polymers involves conformational alterations of the IIb/IIIa receptors, it is tempting to speculate that steric rearrangement of receptors in the platelet membrane may alter associations between the cell membrane and cytoskeletal elements, whether by generation of elevated cytoplasmic calcium ion concentration,[51] production of diacyglycerol,[79] or via some other signal, which in turn promotes the release of the contents of various cytoplasmic granules. Alternatively, interaction of fibrinogen polymers with the platelet might activate the cell by a cumulative effect on cytoplasmic calcium ion concentration. It is possible that multiple GP IIb/IIIa complexes, operating as calcium channels,[80] might lead to an elevation of platelet-free [Ca^{2+}] sufficient to activate the cell's internal excitatory processes.

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HLA-DR–Associated Invariant Chain Is Highly Expressed in Chronic Lymphocytic Leukemia

By Franco Narni, Jiro Kudo, Wendy Mars, Bruno Calabretta, Dagne L. Florine, Bart Barlogie, and Grady F. Saunders

Total RNA extracted from peripheral blood lymphocytes of a patient with B-cell chronic lymphocytic leukemia (CLL) and the poly (A^+) RNA was purified. A cDNA library was constructed and approximately 4,000 clones were screened in order to identify genes preferentially expressed in CLL. A relatively low repetition frequency characterizes the majority of the abundant mRNA species present in CLL lymphocytes. One clone, corresponding to the mRNA encoding the HLA-DR–associated invariant chain, was selected and its expression was examined in different leukemic cell populations and in normal tissues. DNA-RNA hybridization studies showed that the invariant chain mRNA (In-mRNA) is detectable in RNA preparations from human blood cells and their precursors, whereas no In-mRNA is found in several other tissues examined. Among various normal and leukemic leukocyte populations, the highest levels of In-mRNA are found in CLL. Therefore, a role of In-chain mRNA as a marker of CLL is proposed. Our data support a relationship between high levels of invariant chain mRNA and the out of cycle condition of CLL peripheral blood lymphocytes.

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LEUKEMIAS vary with regard to their natural disease course, the pattern of organ involvement, and the responsiveness to chemotherapy. Such differences are likely to reflect the biological features of the cell type involved. Thus, analysis of leukemic cells at the molecular level may help elucidate the mechanisms underlying the biological features peculiar to the different forms of leukemia. Currently, intensive investigation focuses on cellular genes, the abnormal activation of which might play a role in neoplastic transformation.[1] Transforming sequences (oncogenes) identified by transfection experiments can be both tissue specific and, within a given tissue, stage specific.[2] In some B-lymphocyte neoplasms, the c-myc oncogene appears to be activated by juxtaposition to immunoglobulin gene regulatory sequences.[3] Furthermore, in somatic cell hybrids, the translocated myc oncogene of Burkitt's lymphoma is active in myeloma cells but is suppressed in lymphoblastoid cells.[4] These observations suggest that oncogenes must interact with genes whose function is relevant to the normal differentiation pathways of the target cells. If this is indeed the case, it is important to identify genes that are active in leukemic cells pertaining to different lineages. Certain leukemic populations, as they occur in vivo, represent the expansion of a clone of transformed cells that are relatively homogenous with regard to differentiation and stage of maturation. Construction of cDNA libraries, which are representative of the mRNA species in a cell population, and comparative screening of such libraries allows the identification of genes that are preferentially expressed in selected leukemic populations.[5-7]

In the heterogenous group of disorders classified as chronic B-cell leukemias, some species entities like prolymphocytic leukemia and hairy cell leukemia have been identified. The vast majority of patients with chronic B-cell leukemia suffer from B-CLL, a disease characterized by the slow proliferation of a clone of mature-appearing lymphocytes.[8-10] We extracted RNA from peripheral blood lymphocytes of a patient with B-CLL and constructed a cDNA library. To identify sequences preferentially expressed in CLL, the library was screened with ^{32}P-cDNA synthesized from CLL lymphocytes and CML granulocytes. Three copies of a cDNA clone corresponding to the messenger RNA encoding the HLA-DR–associated invariant chain (In-mRNA) were isolated.[11] The levels of In-mRNA were then analyzed in peripheral blood from 48 patients with various forms of leukemia as well as in bone marrow and peripheral blood leukocytes and in a number of normal tissues of normal donors.

MATERIALS AND METHODS

Cells. Peripheral blood leukocytes from leukemic patients were obtained following leukapheresis in accordance with institutional guidelines. The characterization of the leukemic cell populations included morphologic examination, cytochemical reactions (PAS, Sudan black myeloperoxidase, chloroacetate esterase, nonspecific esterase), surface markers (Sm Ig, E-rosettes receptor, Ia-like antigens, B-cell antigen B-1, Leu-1 antigen, monocytic antigen M-1, cALLa), and intracellular enzyme analysis (Tdt). Also, the percentage of S-phase cells in bone marrow specimens was determined by flow cytometry.[12] Typically leukemic cells accounted for at least 80% of the nucleated cells in the blood and bone marrow. Our studies were performed with samples from both untreated and previously treated patients.

Heparinized peripheral blood from normal donors was obtained by venipuncture, and leukocytes were collected after erythrocyte sedimentation. Mononuclear cell enrichment was achieved by Ficoll-Hypaque density gradient centrifugation. Mixed lymphocyte cultures were established in RPMI 1640 medium (Gibco) supplemented with 20% fetal calf serum (Irvine Scientific) in the presence of PHA (Wellcome) 200 μ/mL, at 37 °C in 5% CO_2. Granulocytes were recovered from the pellet of the Ficoll-Hypaque gradient after lysis of residual red blood cells in 0.144 M NH_4Cl, 0.017 mol/L Tris-HCl, pH 7.2. Normal B lymphocytes were purified from the mononuclear cell fraction after removal of monocytes through adherence to plastic tissue culture flasks and disposal of E-rosette–forming cells.

Stimulation of CLL lymphocytes was performed in Ham's F-10 Medium (Gibco) supplemented with 20% FCS. Lipopolysaccharide

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B (*Escherichia coli* 055:B5, Difco) was added to the culture at final concentration of 100 µg/mL.

Bone marrow was obtained from normal donors following aspiration from the iliac crest. Spleen, pancreas, liver, kidney, gut, and muscle were autoptic fragments from a patient with bladder carcinoma. No metastatic tumor was present in the examined tissues.

cDNA library construction and screening. The cDNA library was constructed essentially as described by Land et al.[13] Briefly, poly(A$^+$)RNA was separated from total RNA after three passages over oligo-dT cellulose (Collaborative Research).[14] Single-stranded cDNA was synthesized with avian reverse transcriptase (Seikagaku America, Inc), and double-stranded cDNA was synthesized with *E. coli* DNA polymerase I (Boehringer Mannheim). After addition of deoxycytidine homopolymer tails, cDNA was cloned into the *Pst* I site of deoxyguanosine tailed plasmid pBR322. Recombinant plasmids were transfected into *E. coli* K12 (strain RR1). After spreading on L-broth/bacto-agar plates containing tetracycline, 4,000 individual colonies were transferred to microtiter plates. The screening was performed essentially as described by Grunstein and Hogness,[15] using ^{32}P-labeled cDNA transcripts[16] of poly(A$^+$)RNA extracted from CLL and CML cells as probes.

Hybridizations. Cells were washed twice in phosphate-buffered saline and total RNA was extracted as described by Frazier et al.[17] In Northern blot experiments 10 to 20 µg of total RNA or 2 µg of poly(A$^+$)RNA were denatured[18] by one-hour incubation at 50 °C in 50% DMSO, 1 mol/L glyoxal, and 10 mmol/L phosphate buffer, pH 7. After electrophoresis through a 1% agarose gel, RNA was transferred to nitrocellulose paper presoaked in $20 \times$ SSC ($1 \times$ SSC = 0.15 mol/L NaCl, 0.015 mol/L Na$_3$ citrate, pH 7) and filters were baked for two hours at 80 °C under vacuum.[19] After hybridization to In-cDNA, the integrity of RNA in each lane was confirmed by hybridizing the same filters to a probe containing 28S ribosomal DNA sequences.

In dot-blot hybridizations RNA was heated to 50 °C in 1 mol/L glyoxal, 10 mmol/L phosphate buffer, pH 7, for one hour and chilled. Serial dilutions were made in $10 \times$ SSC and 4 µL aliquots were spotted onto nitrocellulose. Cytoplasmic dot hybridizations were performed as described,[7] with a modification of the method of White and Bancroft.[20]

Several clones corresponding to invariant chain mRNA were isolated in our laboratory. In most hybridization experiments, however, we used clone p7-2D-1446[21] as a probe, labeled to a specific activity of 2×10^8 cpm/µg. In order to determine the abundance of In-chain mRNA in CLL, 7-2D-1446 insert was released by digestion of the recombinant plasmid with *Pst* I and purified after separation on a 4% polyacrylamide gel. Known amounts of the purified insert and of CLL poly(A$^+$)RNA were immobilized to nitrocellulose using a minifold II aparatus (Schleicher and Schuell). The filter was hybridized to radiolabeled p7-2D-1446 insert.

All hybridization reactions were performed in $6 \times$ SSC, 10 mmol/L EDTA, $5 \times$ Denhardt's solution (Denhardt's solution = 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.5% SDS, 200 µg/mL sonicated salmon sperm DNA at 68 °C for 12 to 24 hours. After hybridization filters were washed in $0.2 \times$ SSC, 0.5% SDS at 68 °C for six hours. Filters were exposed at −70 °C, with intensifying screens. Scanning densitometry of autoradiographic films was performed using a quick scan instrument (Helena Laboratories).

RESULTS

^{32}P-labelled cDNA transcribed from CLL and CML poly(A$^+$)RNA was hybridized to 4,000 colonies from a CLL cDNA library. After the first screening, most of the clones appeared to represent RNAs expressed at comparable levels in both cell populations, and several recombinants appeared to contain sequences even more highly represented in CML granulocytes. Fifty clones, hybridizing to mRNA species apparently more abundant in CLL poly(A$^+$)RNA, were selected and filters were hybridized to cDNA from three different patients with CLL and three different patients with CML. Only three of the clones that had been selected for the second screening appeared to be reproducibly more highly represented in RNA extracted from CLL lymphocytes. The purified inserts cross-hybridized under conditions of high stringency, indicating that the three clones corresponded to the same mRNA species. A computer search of the nucleotide sequence data bank identified this sequence as the messenger RNA encoding the HLA-DR–associated invariant chain.[21]

In filter hybridization reactions the amount of nucleic acid bound to nitrocellulose is critical. To overcome the potential problem represented by unequal growth of individual colonies during the initial screening procedure, constant amounts of poly (A$^+$)RNA or total RNA extracted from different cell populations were spotted onto nitrocellulose filters and the higher abundance of In-mRNA in CLL lymphocytes was confirmed after hybridization with the radiolabeled cDNA (Fig 1). To ensure that the observed differences were due to specific hybridization of our probe with a discrete RNA species and to determine the size of the corresponding mRNA, Northern blot hybridization was performed. Total RNA was extracted from peripheral blood leukocytes of 6 patients with CLL, 5 patients with CML in chronic phase, 2 patients with CML in blast crisis, 4 patients with ALL, and 6 patients with AML. All of the CLL populations examined were of the B-cell type and were positive for Sm Ig, B-1

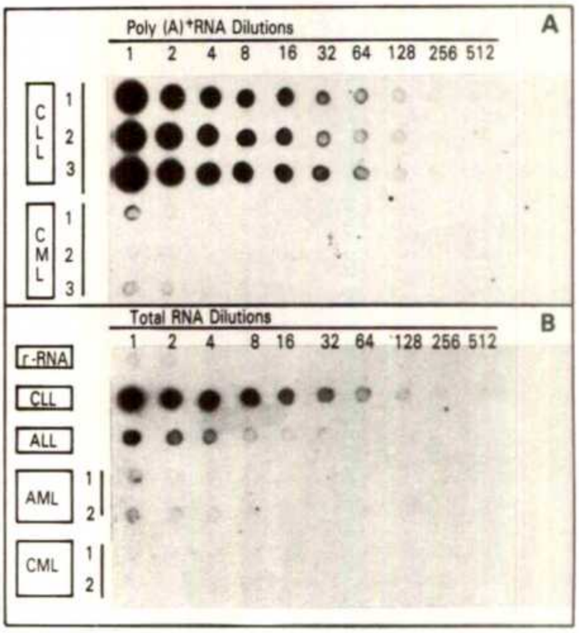

Fig 1. Dot blot hybridization analysis of In-mRNA in different leukemic populations. Two µg of poly(A$^+$)RNA (A) or 10 µg of total RNA (B) and serial dilutions were spotted onto nitrocellulose and hybridized to ^{32}P-7-2D-1446. rRNA = human ribosomal RNA separated after three passages on oligo(dT)cellulose in 20 mmol/L Tris, pH 7.6, 0.5 mol/L NaCl, 1 mmol/L EDTA, 0.1% SDS.

antigen, and Ia-like antigens. The percentage of cells in S phase in the bone marrow from the same patients ranged from 15% to 20% in CML samples and from 1% to 2% in CLL. Intermediate values were observed in samples from patients with acute leukemia. RNA was also extracted from bone marrow and peripheral blood leukocytes of three normal donors. A single band of approximately 1.5 kb was detected in all cases examined; however, a wide variability in the levels of In-mRNA was observed. The intensity of the observed bands was compared by scanning densitometry and integration of the peak areas (Fig 2). Very low signals were found in the bone marrow of normal donors and of patients with CML in chronic phase and in some cases of AML. Intermediate signals were observed in normal peripheral blood leukocytes, some cases of AML, and in most patients with ALL. High hybridization signals were typical of CLL but were also found in one case each of lymphoid blast crisis of CML (cALLa +) and ALL. The difference between the lowest detectable level and the highest signal intensity was about 100-fold. The intensity of the observed bands, expressed in arbitrary units, ranged in CLL from 10.4 to 19.2 (mean 14.11), in ALL from 2.1 to 8.4 (mean 4.37), in AML from 0.2 to 2.1 (mean 0.88), and in CML in chronic phase from 0.3 to 2.1 (mean 0.88). The values observed in normal cells varied in peripheral blood from 2.2 to 3.3 (mean 2.63) and in bone marrow from 0.2 to 1.2 (mean 0.60). The ratio between the calculated mean values shows that in CLL the level of In-mRNA is approximately 16-fold higher than in myeloid leukemias and three-fold higher than in ALL. When the levels observed in CLL samples were compared to the values observed in normal peripheral blood mononuclear cells and normal bone marrow cells, ratios of 5.36 and 23.5, respectively, were observed.

In dot-blot and Northern-blot hybridization experiments, a constant amount of RNA from different sources was analyzed, thus permitting quantitation of the fraction of In-RNA present. Total RNA content varies in different cell types and is particularly low in lymphocytes.[22,23] Therefore, to compare the amounts of In-mRNA in different cell populations regardless of total RNA content, a constant number of cells from 23 different patients (6 CLL, 11 CML, 4 AML, and 2 ALL) was analyzed using a minor variation[7] of the cytodot technique described by White and Bancroft.[20] Using this method the expression of In-mRNA was again found to be higher in CLL cells than in samples from patients with other forms of leukemia (data not shown).

In-mRNA expression was also examined in several normal tissues and subpopulations of normal leukocytes. In-mRNA could not be detected in either total RNA or poly(A$^+$)RNA preparations from normal placenta (3 different specimens), liver, pancreas, gut, and muscle. Furthermore, we did not observe hybridization of our probe with RNA from 2 normal kidney specimens. In normal leukocytes, In-mRNA was most strongly expressed in peripheral blood B lymphocytes, whereas normal spleen tissue failed to show detectable hybridization signals (Fig 3). RNA extracted from granulocytes produced a faint band only after prolonged exposure (data not shown); however, approximately 10% mononuclear cells remained in the granulocyte-enriched fraction.

Mixed cultures of peripheral blood mononuclear cells from two normal donors were established in the presence of PHA, and RNA was extracted at 0, 6, 24, 48, and 60 hours. Autoradiography performed on aliquots of the same cultures incubated with [^{3}H]-methylthymidine showed that, after 60 hours, approximately 60% of the cells were in S phase. Additionally, the highest expression of histone H3 gene, whose transcriptional activation occurs during the S phase of the cell cycle,[24] was observed at this time point. A decrease in the level of In-mRNA was evident after six hours and

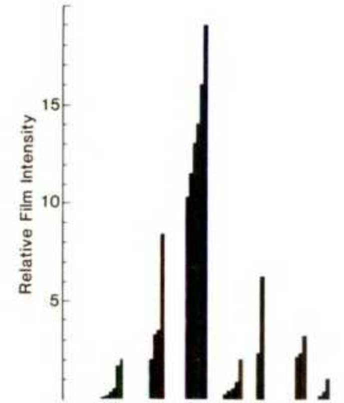

Fig 2. Relative levels of In-mRNA in leukemic and normal leukocytes. The intensity of the bands observed in Northern blot hybridizations was determined by scanning densitometry of the films. The values obtained after integration of the peak areas, expressed in arbitrary units, are reported in the histogram. To compare the results obtained in different experiments at least one RNA sample was included in each blot as a reference. Each sample was analyzed in at least two different Northern blots. BC, blast crisis of CML; PB, peripheral blood leukocytes; BM, bone marrow.

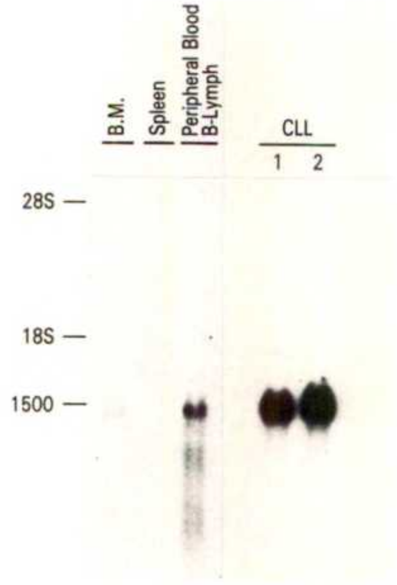

Fig 3. Northern blot analysis of In-mRNA in normal bone marrow, spleen tissue, and peripheral blood B lymphocytes. Ten μg of total RNA were loaded in each lane. Two CLL RNA samples were also included.

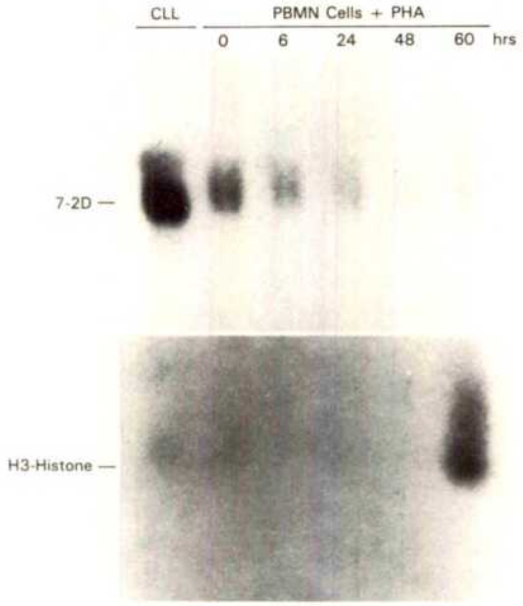

Fig 4. Effect of PHA stimulation on the levels of In-mRNA in peripheral blood mononuclear cells (PBMN cells) from normal donors. Twenty μg of RNA extracted before adding PHA and at different times after stimulation were loaded per each lane. The same filter was hybridized to 7-2D-1446 probe (upper panel) and, subsequently, to a probe corresponding to histone the H3 gene (lower panel).

In-mRNA was almost undetectable after 60 hours. (Fig 4). This observation showed that the reduction in In-mRNA was associated with heterogeneous cell proliferation. The high In-mRNA levels in CLL could be related to the high percentage of nondividing B cells characteristic of this disorder. To test this hypothesis, cells from a patient with CLL were cultured in the presence of LPS and RNA was extracted before adding this mitogen and after two, three, four, and five days of stimulation. We indeed observed a progressive reduction in In-mRNA levels (Fig 5), which was accompanied by an increase of myc-mRNA (data not shown). There was no variation in levels of In-mRNA when cells of the same patient were cultured in the absence of LPS.

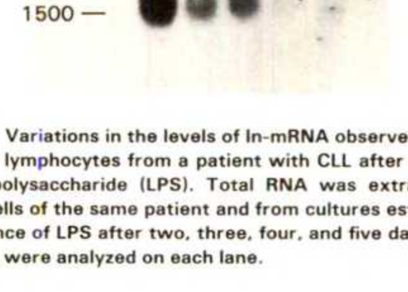

Fig 5. Variations in the levels of In-mRNA observed in peripheral blood lymphocytes from a patient with CLL after stimulation with lipopolysaccharide (LPS). Total RNA was extracted from primary cells of the same patient and from cultures established in the presence of LPS after two, three, four, and five days. Twenty μg of RNA were analyzed on each lane.

The percentage of In-chain mRNA in poly(A^+)RNA extracted from CLL lymphocytes was estimated as described in the Materials and Methods section. Five μg of poly(A^+)RNA gave a signal corresponding to 1 ng of purified 7-2D-1446 insert (Fig 6). Since the poly(A^+)RNA is single-stranded and the insert is double-stranded, and considering that approximately 50% of the RNA in the poly(A^+)RNA–enriched preparation was represented by ribosomal RNA, we estimate that In-mRNA ranges from 0.05% to 0.10% by weight of the polyadenylated sequences present in CLL cells.

DISCUSSION

Our initial objective was to identify, among abundant mRNA species, sequences whose expression was relevant to the biological characteristics of B-CLL. In planning the screening of our cDNA library, CML appeared to be an appropriate counterpart because granulopoiesis is represented at all stages of maturation in the chronic phase of CML.

In resting lymphocytes the RNA content is lower than in many other cell types,[22,23] and the heterogeneous nuclear RNA is mainly confined to the high molecular weight region.[25,26] Complexity and abundance of the polyadenylated RNA, as deduced by hybridization kinetics, reveal a decreased representation of the middle-repetitive sequences and fewer copies of the more abundant components.[27] These

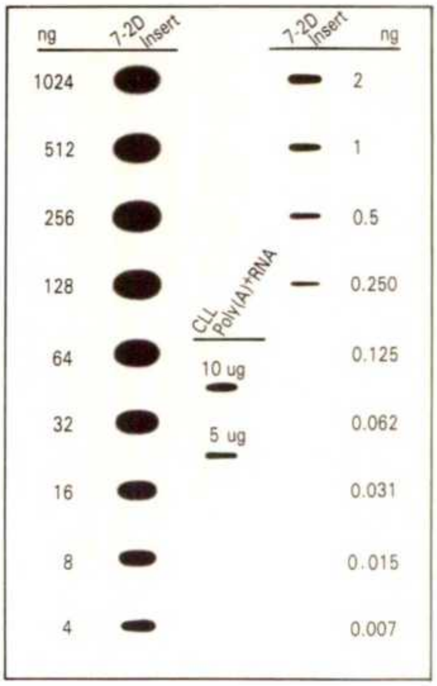

Fig 6. Abundance of In-mRNA in CLL lymphocytes. The 7-2D-1446 insert was denatured at 100 °C for 10 minutes and serially diluted. CLL poly(A^+)RNA was heated to 50 °C in 1 mol/L glyoxal, 10 mmol/L phosphate buffer, pH 7. Prior to application each sample was adjusted to 10 × SSC.

findings are consistent with the lack of specialized functions and the low metabolic activity characterizing the lymphatic cell at this maturation stage. Although the kinetics of hybridization can show the general composition of different sequences transcribed in a cell population, the construction and screening of cDNA libraries allows a more detailed analysis, since each sequence can be individually monitored. Our studies, while they confirm that a general paucity of specific abundant mRNA species is a distinctive feature of the resting lymphocyte, provide identification of at least one moderately abundant sequence in lymphocytes.

This sequence corresponds to the mRNA encoding the HLA-DR–associated invariant protein.[21] The expression of the In-chain gene seems to be restricted to hematopoietic tissues. Although variable amounts of In-mRNA are found in different types of blood cells, high levels are characteristic of B lymphocytes. As determined by Northern blot hybridization, the level of In-mRNA is several-fold higher in CLL than in myeloid leukemias. Levels of In-mRNA approaching those observed in CLL have been found only in one of four cases of ALL and in one lymphoid blast crisis of CML.

In both normal and CLL lymphocytes, the level of In-mRNA decreases following mitogen stimulation. Although a role of the invariant chain in the assembly and transport of α and β HLA-DR chains has been proposed,[28,29] the function of this protein has not been established. A correlation between the expression of this gene and cell quiescence has been demonstrated in different cell types[30,31] and a possible role in the control of cell proliferation has also been considered.[32] While a role of the HLA-DR invariant chain in the control of cell proliferation can not be inferred, our studies demonstrate an inverse relationship between the expression of the In-gene and cell proliferative activity. Thus, high levels of In-messenger in CLL are consistent with the "accumulative" nature of cells in this disorder with low proliferative activity.[33-35] Levels of In-mRNA expression exceeding those observed in normal lymphocytes are consistent with the "deep G_0" state attributed to CLL lymphocytes, whose low responsiveness to various mitogens has been reported.[36,37]

The expression of In-mRNA is, as a rule, synchronous with the expression of class II histocompatibility antigens.[30] Therefore, the pattern of In-mRNA expression in PHA-stimulated mononuclear cells is at present unclear. Since most T lymphocytes are known to be Ia negative, the basal level of In-mRNA and the subsequent variations observed after stimulation might be related to the B lymphocytes and/or monocytes present in the cultures.

We failed to detect In-mRNA in 10 μg of total RNA extracted from normal human spleen tissue, which is in contrast to In-chain expression by murine splenic B lymphocytes.[11] The absence or low expression of In-mRNA in human spleen may have several explanations. First, RNA was extracted from total splenic tissue obtained two hours after the patient's death and frozen at −70 °C and B lymphocytes were not separated from the other cell types. Second, splenic B lymphocytes are heterogeneous, and differences in the pattern of In-mRNA expression in different B-lymphocyte subsets might exist. Third, the spleen tissue was obtained from a patient with bladder carcinoma who had received chemotherapy 1 month before death. At the time of death, the patient was still markedly leukopenic, suggesting that chemotherapy might have depleted the spleen of recirculating B lymphocytes. Finally, the observed difference in In-mRNA expression may simply reflect a phenomenon relating to species specificity. Although the availability of human tissues is a limiting factor, more experiments are necessary to study this problem.

At the time of our study, we were unaware that our isolated clones corresponded to the invariant chain mRNA. Therefore, our results can be regarded as the independent isolation of a cDNA clone that is preferentially expressed in CLL, which corresponds to the invariant chain gene. Our findings demonstrate that the construction and comparative screening of cDNA libraries is a useful approach to identifying prominent molecular features of different forms of leukemia. Thus, we conclude that the level of In-chain mRNA provides a new marker that might join previously identified features characteristic of CLL.

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Use of Highly Polymorphic DNA Probes for Genotypic Analysis Following Bone Marrow Transplantation

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Christine Murray, Tak Takvorian, Jerome Ritz, and Helen Donis-Keller

The use of DNA markers known as restriction fragment length polymorphisms is a sensitive and informative method of distinguishing patient and allogeneic donor cells after bone marrow transplantation. To apply the test, it is necessary in each case to find DNA probes that display patient-specific and donor-specific bands in Southern transfer hybridization. We have isolated a set of 12 cloned DNAs from highly polymorphic loci by which siblings can usually be distinguished. With just four of these probes, we can expect to distinguish the genotypes of the recipient and a sibling donor in more than 99% of cases (except between identical twins). The availability of many highly polymorphic probes also allows selection of an optimal probe for each case, one that can detect both the patient and donor-specific bands in a single hybridization with maximum resolution and sensitivity. We have applied these probes to the analysis of cells from peripheral blood and bone marrow after transplantation and demonstrated their usefulness in confirming engraftment of donor cells or graft rejection, and in detecting mixed lympho-hematopoietic chimerism.

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BONE marrow transplantation is becoming the therapy of choice for a number of hematopoietic disorders and malignancies.[1-3] Because of the complex interactions among the host tissues, the donor immune system and the residual host immune system, analysis of the respective contributions of donor and recipient cells to the lympho-hematopoietic system is critical to interpretation of the events following transplantation.

The use of DNA sequence polymorphisms is an informative and versatile method of distinguishing patient and donor cells.[4-8] Phenotypically neutral sequence variations in the population can be identified as restriction fragment length polymorphisms (RFLPs), usually observed in Southern transfer hybridizations with cloned DNA probes.[9] Among different individuals, the restriction fragments homologous to a cloned DNA probe can occasionally differ in length, either because of mutations in restriction enzyme cleavage site or variation in the length of DNA between restriction sites as a result of insertions or deletions. Stably inherited as a part of an individual's genome and propagated to all cells, these DNA polymorphisms constitute a reliable set of markers for cells originating from a particular individual. Unlike other transplantation markers (eg, red blood cell antigens, immunoglobulin isotypes, karyotypes),[10-18] RFLP analysis is applicable to all hematopoietic cells except fully differentiated erythrocytes, in aggregate or in sorted cell fractions.[5,8]

The applicability of genotypic analysis to particular cases depends on discovery of informative DNA polymorphisms, ie, distinguishable alleles in the marrow donor and recipient. The likelihood that a particular probe will reveal distinct alleles in recipient and donor depends on the number of alleles and their distribution in the population. Most RFLPs are two allele variants of a single restriction site and therefore rarely informative in comparisons of patient and donor genotypes. One useful probe is the plasmid pDP34,[19] which hybridizes to distinct bands from the X and Y chromosomes and can always be used to detect the male genotypic pattern in sex mismatch cases. Analysis of cellular origin in most transplantation cases has depended primarily on the cloned DNA probe pAW101,[20] which identifies a locus with multiple RFLP alleles. However, because the donor and recipient of a transplant are siblings or very closely related, testing a single genetic marker, even a highly polymorphic DNA sequence, cannot guarantee that the two cell types can be distinguished. For no matter how many alleles exist in the population, each parent carries at most two different alleles, and two siblings have a 1/4 chance of inheriting the same two alleles of a polymorphic locus from their parents, and an additional 1/2 probability of sharing one parental homologue. This means that siblings will be indistinguishable by any single genetic marker in at least 25% of the cases. Probes for more than one highly polymorphic locus are therefore necessary to ensure detection of distinguishable patient and donor alleles. In this paper we describe a set of probes for highly polymorphic loci in the human genome that virtually guarantees the feasibility of determining the origin of nucleated cells in bone marrow transplants, even between closely related individuals.

The utility of these probes in analysis of transplant cases is demonstrated in this preliminary application to a set of leukemia patients who received bone marrow transplants from related allogeneic donors. Using genotypic analysis we have been able to distinguish patients with stable engraftment of donor cells exclusively, patients with chimeric cell populations associated with leukemic recurrence, and patients who appear to develop a stable chimeric condition.

MATERIALS AND METHODS

Patients. Cells were obtained prior to bone marrow transplantation from 10 patients and their related allogeneic donors. Each of those patients underwent marrow transplantation as treatment for hematologic malignancy. Eight patients received marrow from HLA-compatible siblings and two patients received marrow from partially incompatible related donors. In six instances patients and donors were of the same sex, but in four cases there was a sex mismatch. Transplantation and subsequent sampling of blood and

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bone marrow were approved by the Institutional Review Boards of the Dana-Farber Cancer Institute and Brigham and Women's Hospital and were performed with the informed consent of the patients.

Cell samples. Peripheral blood and bone marrow samples from patients and donors were collected in preservative-free heparin and mononuclear cells isolated by Ficoll/Hypaque density sedimentation. In some instances, peripheral blood mononuclear cells were cultured in vitro following stimulation with either phytohemagglutinin (PHA) or allogeneic B-cell line (Laz 388). After this initial stimulation, cells were cultured in lymphocyte conditioned medium (LCM) containing IL-2. All cell samples were cryopreserved in 10% DMSO using standard techniques and stored in the vapor phase of liquid nitrogen.

Cellular DNA preparation. High molecular weight DNA was prepared from isolated nuclei obtained by centrifugation following solubilization of the cell suspension in Triton X-100. The nuclear pellet was resuspended in 1% SDS and treated with Proteinase K (Boehringer/Mannheim), 10 μg/mL, for 16 hours at 42 °C. After extraction with phenol and chloroform/isoamyl alcohol (24:1), DNA was precipitated with isopropanol (1.5 vol), redissolved in TE (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA), and precipitated with ethanol. DNA was redissolved in TE at a concentration of 250 μg/mL. Recovery of DNA from frozen cell samples was generally 5 micrograms per 10^6 cells.

Analysis of genomic restriction fragments. DNA samples at 175 μg/mL were digested to completion with restriction endonucleases (5 U/μg) from New England Biolabs, Beverly, Mass, in conditions recommended by the supplier. The extent of digestion was monitored in identical reaction mixtures containing both 1 μg of human DNA and 1 μg bacteriophage lambda DNA, stained with ethidium bromide after agarose gel electrophoresis; complete digestion was assumed if the lambda DNA band pattern represented a limit digest. Digested DNA samples were size fractionated by electrophoresis in 0.8% agarose gels, transferred to nylon membranes (Zeta-Bind, AMF Cuno, Meriden, Conn) in 25 mmol/L sodium phosphate, pH 6.5, according to Barker et al.[21] After prehybridizing the filters for 2 to 24 hours at 42 °C in 5 × SSC, 40 mmol/L phosphate, pH 7, 5 × Denhardt's solution, 100 μg/mL denatured salmon DNA, 10% dextran sulfate, and 50% formamide, ^{32}P-labeled probe was added (10^6 dpm/mL) to hybridize for 20 hours at 42 °C. After hybridization, filters were washed for 30 minutes at 20 °C with 2 × SSC, 0.1% SDS, and 60 minutes at 65 °C in 0.1 × SSC, 0.5% SDS. Autoradiography was carried out for one to five days at −70 °C with Kodak XAR-5 film and intensifying screens (DuPont Cronex Lightning-Plus).

Probes from polymorphic loci. These polymorphic DNA probes were isolated and characterized at Collaborative Research, Inc, for application to genetic linkage mapping.[22,23] Human genomic clones in bacteriophage Charon 4A were isolated from the library of Lawn et al.[24] Randomly chosen, single-copy phage clones were screened in Southern blot hybridizations to restriction digests from five individuals for restriction fragment length polymorphisms.[22] Twelve genomic clones with multiple allelic variations suitable for genotypic analysis of bone marrow transplants were selected for this study.

Recombinant bacteriophage DNA was prepared from plate lysates by the methods of Davis et al[25] and radioactively labeled by nick translation[26] with ^{32}P deoxynucleotide triphosphate (New England Nuclear, Boston) to a specific activity of 1 to 5 × 10^8 dpm/μg.

All work with recombinant bacteriophage was carried out under P1 conditions of containment in accordance with National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

RESULTS

Characterization of highly polymorphic DNA probes. We have obtained from a human genomic library a set of DNA clones that hybridize to sequences with extraordinary polymorphic variation.[23] Hybridization patterns of these probes to digests of DNA from a series of unrelated people show a spectrum of variable bands, with few individuals sharing the same pattern (Fig 1). The probes are randomly cloned single-copy sequences ranging in length from 11 to 17 kilobases inserted in the bacteriophage Charon 4A vector. Because the cloned DNA segments are large, the probes generally hybridize to several contiguous restriction fragments in the genomic digests. Each probe hybridizes to restriction fragments derived from a single chromosomal locus, as seen from the Mendelian inheritance pattern of the RFLPs in human pedigrees (data not shown).

For most of the probes described here, the same restriction fragment length variation among individuals can be detected with several different restriction enzymes, suggesting that the polymorphisms result from DNA sequence rearrangements at these loci rather than simple mutation of restriction sites. At some loci (detected with LAM4-159, LAM4-427, LAM-123, and LAM4-1214, for example), several different restriction fragments vary in length independently, with the result that each of the two alleles carried by an individual consists of multiple fragments potentially different from those of another individual. Fig 2 displays the allelic sets of PstI restriction fragments from the locus hybridizing to LAM4-159 as observed in chromosomes of 18 unrelated persons. Each locus consists of variable segments hybridized by the left side of the probe and by the right side of the probe, separated by fragments of constant size in the center. Each of the eight known variants of the left-side PstI fragment is associated with more than one of the eight observed right-side variants in different chromosomes, generating at least 16 allelic combinations already observed at this locus.

Not only does each of these loci exist in a large number of allelic forms, but the alleles are also dispersed in the population such that the probability that two parents will share the same alleles is low. This probability is typically expressed as the polymorphism information content, or PIC,[9] which is calculated from the allele frequencies and is a measure of the probability that the parental chromosome contributions can be determined in the offspring. Table 1 lists the estimated PIC values for each of our polymorphic probes; most are at least as informative as pAW101 (PIC = 0.85), and more informative than all but a few of the known polymorphic markers in the human genome.[9,27] From the number of alleles observed at each locus and their frequencies in the population, we can also calculate the likelihood that each probe will be informative in the special case of comparing genotypes of two siblings (Table 1). For the probes with the highest PIC values, the predicted probability of distinguishing siblings approaches the theoretical maximum of 75%.

Because the occurrence of distinguishable alleles at one locus is independent of the alleles at another locus, the application of the test can be extended to virtually all patient donor pairs by selecting from a set of highly polymorphic

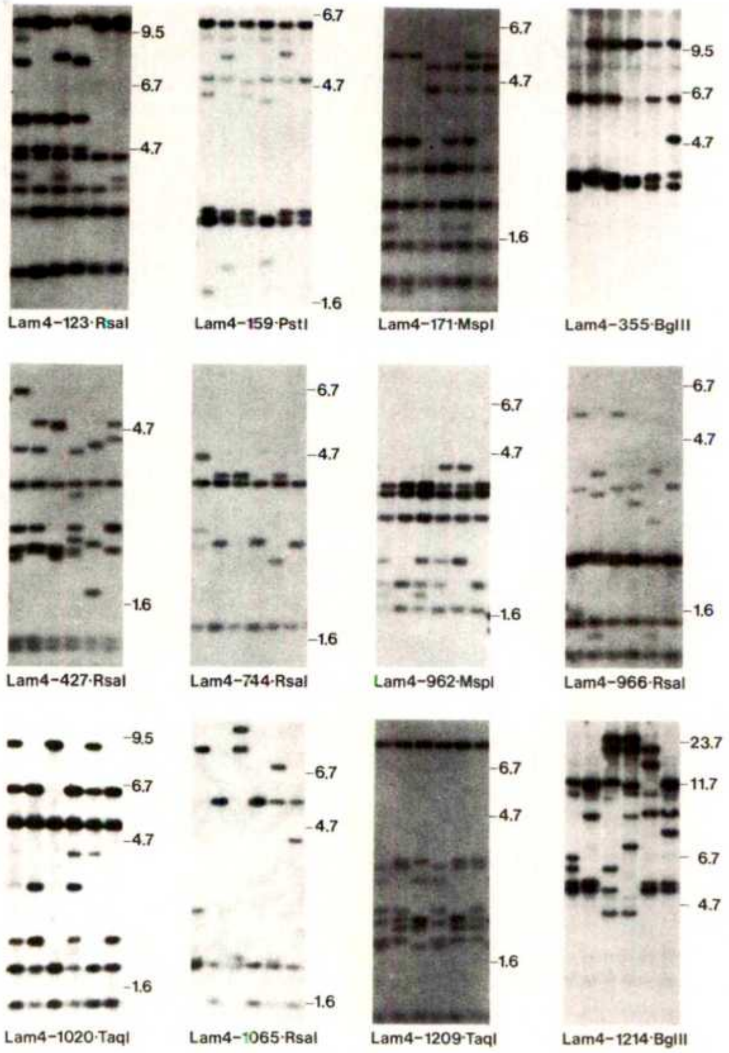

Fig 1. Restriction fragment patterns at highly polymorphic loci. DNA from 6 unrelated individuals was digested with restriction enzymes shown and hybridized to each cloned DNA probe as described in Materials and Methods. Migration of restriction fragment markers of known molecular weight is shown at right of each autoradiogram.

probes. In comparing sibling donor and recipient genotypes, this premise is valid if the polymorphic loci are not genetically linked, ie, if they are on different chromosomes or are separated with a recombination frequency approaching 50%. (A patient and donor who have inherited the same marker from a parent are also likely to have inherited the same DNA sequences in the surrounding region of that chromosome.) We have traced the inheritance of alleles of each of the polymorphic probes listed in Table 1 in several large, multigenerational families and compared their inheritance patterns for indication of genetic linkage. None of the probes is closely linked to any other with measured recombination frequency less than 25% (unpublished results). Application of each RFLP probe to a patient-donor pair therefore represents an independent test for genetic heterogeneity. The probability of genetic identity at all loci tested is therefore the product of the probability of identical alleles with the individual probes (Table 1). With all of the 12 probes used in this study, the probability that two siblings would be identical is only 1.3×10^{-6}. In fact, we can be quite confident of distinguishing siblings with only four of the most polymorphic probes (LAM4-427, 1214, 355, and 159). Genotypic comparisons at just these loci would be informative in more than 99% of the cases.

Identification of informative probes in transplant cases. Genotypic analysis of cellular DNA in bone marrow transplantation proceeds in two stages. First, DNA samples of donor and recipient origin are tested with probes from highly polymorphic loci to determine for each locus whether the two genotypes are distinguishable in Southern transfer-hybridization. It is necessary to find both donor and patient-specific bands in the hybridization patterns, so that contributions of both genomes can be distinguished in a mixture of the two. The ideal probe would hybridize to both donor-specific and patient-specific bands in the same restriction digest, so that both genotypes could be detected in the same sample and hybridization.

Probes that fulfill these criteria for informative and efficient genotypic analysis were selected from the panel to apply to ten bone marrow transplant cases. First, the genotypes of the ten transplant recipients and their respective donors were compared by Southern blot hybridization patterns of the 12 probes listed in Table 1. Restriction fragment patterns, such as those shown in the autoradiograms in Fig 3,

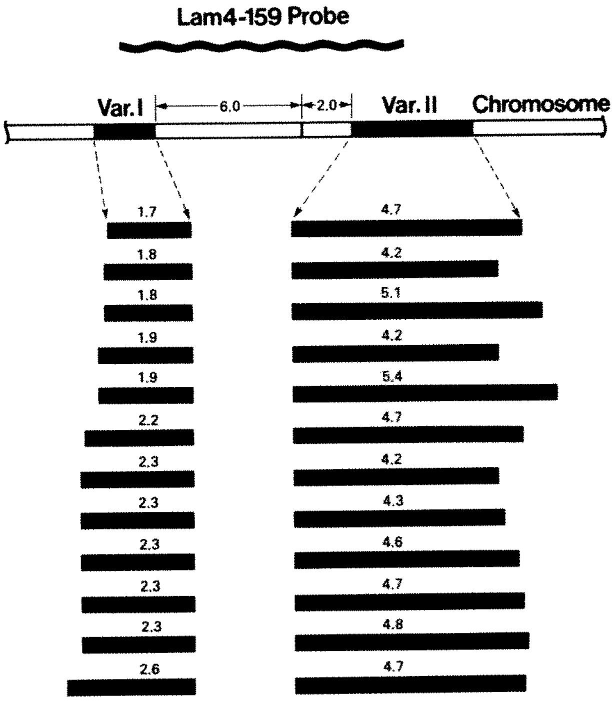

Fig 2. Variable fragment lengths at locus defined by LAM4-159. Four contiguous PstI fragments are detected with the phage clone LAM4-159, two fragments of constant length (6 kb and 2 kb) flanked by two fragments of varying length in different alleles. The length variation in fragment I (Var.I) is apparently independent of the variation in fragment II (Var.II), so that the number of different combinations is large. The examples of LAM4-159 alleles shown are 12 different fragment combinations observed among 36 chromosomes of 18 unrelated individuals.

were scored for the presence of patient-specific and donor-specific bands. As expected, each patient and donor genotype could be distinguished by restriction fragments at several of the probed loci (Table 2). Because of the highly polymorphic character of the genetic loci tested, each probe was informative for several patient-donor pairs. In general, the probes with the highest PIC values were the most successful at distinguishing between patient and donor genotypes. Having identified several informative probes in each transplant case, we chose one probe that was expected to provide the most sensitive and reliable measurement of proportions of each genotype in posttransplant samples. Sensitivity is maximal when bands to be detected show strong hybridization signals, are well separated from other bands, and background hybridization is low. Furthermore, some probes hybridize to more than one patient-specific or donor-specific band in a single digest, providing opportunities for repeated measurement of the proportions of the two DNAs in the same sample (eg, Fig 3A, patient-donor pair #6).

Analysis of cell populations after transplantation. Cell samples taken after bone marrow transplantation were analyzed with the polymorphic probes chosen for each patient-donor pair. Hybridization patterns of posttransplant DNA samples were compared with the patterns of pure donor and recipient DNA in adjacent lanes (Figs 4 and 5). The fraction of the total cell population represented by either donor cells or patient cells was estimated from the respective band intensities in the autoradiogram. Adjacent control lanes contained admixtures of known proportions of patient and donor DNA and served as standards for making quantitative estimates of these DNAs in posttransplant samples. Direct comparison of patient and donor bands in the same hybridization is critical to quantitative measurement of the proportions of the two genotypes, because the intensity of bands can vary substantially depending on the efficiency of transfer of the DNA to the filter, the specific activity of the radioactive probe, and the stringency of hybridization and washing. In particular, it is important to establish the sensitivity of the assay when the autoradiogram shows only the donor genotypic pattern in order to determine the maximum level of cells of patient origin that could have escaped detection. In these hybridizations with the recombinant bacteriophage DNAs as probes, we could detect cellular DNA that was present as 5% to 10% of the total. Elimination of the lambda vector sequences from the same probes can improve the sensitivity to approximately 1% (results not shown).

Results demonstrating successful engraftment of donor cells in patient #1 are shown in Fig 4A. DNA was prepared from peripheral blood taken at 16 to 20 months after transplant, as well as from bone marrow aspirate collected 17 months posttransplant. After restriction enzyme digestion and transfer hybridization with probe LAM4-1214, the restriction fragment patterns of these DNA samples were compared with the pretransplant patient and donor patterns. The presence of the 25 kb, 9.3 kb, and 6 kb bands of donor genotype and the absence of 7.5 kb and 5.6 kb bands characteristic of patient genotype confirms the successful

Table 1. Polymorphic DNA Probes and Their Expected Information Value*

Probe	Enzyme	PIC	Probability Siblings Distinguishable
LAM4-427	RsaI	0.95	0.72
LAM4-1214	BglII	0.94	0.72
LAM4-355	BglII	0.90	0.70
LAM4-159	PstI	0.90	0.70
LAM4-966	RsaI	0.88	0.69
LAM4-123	RsaI	0.81	0.65
LAM4-744	RsaI	0.80	0.65
LAM4-1020	TaqI	0.80	0.65
LAM4-1065	RsaI	0.75	0.62
LAM4-1209	TaqI	0.75	0.62
LAM4-171	MspI	0.70	0.60
LAM4-962	MspI	0.70	0.60

*PIC is the polymorphism information content, the probability of informative segregation of alleles in a given family at the marker locus.[16] The probability that two siblings will be identical at the locus defined by each probe is also calculated from the allele frequencies in the population:

$$\text{Probability of sibling identity} = \frac{1}{4} + \frac{1}{2}\sum_{i=1}^{n} f_i^2 + \frac{1}{2}\sum_{i=1}^{n}\sum_{j=1}^{n} f_i^2 f_j^2 \approx \frac{1}{4} + \frac{1}{2}\sum_{i=1}^{n} f_i^2$$

where f_i and f_j are the frequencies in the population of alleles i and j, and n is the total number of alleles. The probability that the probe will distinguish two siblings is then one minus the probability that they have the same two alleles.

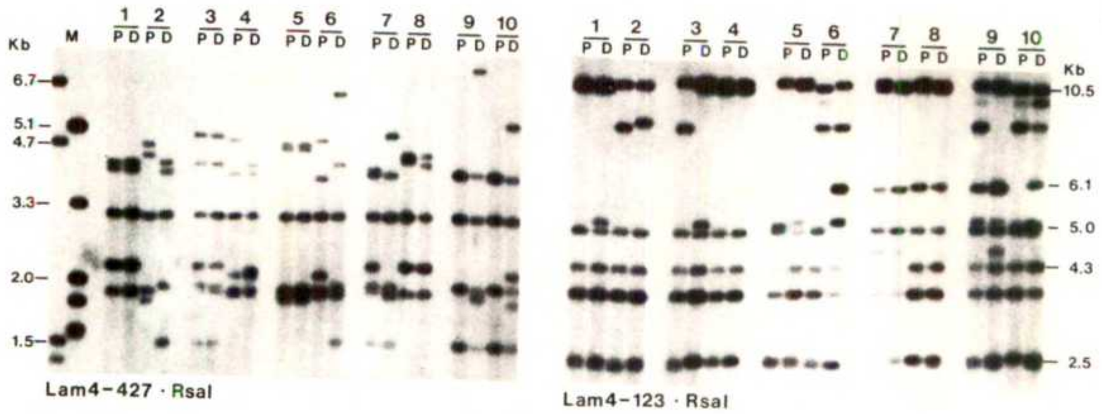

Fig 3. Identification of patient and donor-specific bands in DNA hybridization before transplantation. Pretransplant genomic DNA samples (2 μg) from 10 patient/donor pairs were digested with RsaI and hybridized with RFLP probes LAM4-427 and LAM4-123. Patient and donor-specific fragments can be detected in all 10 cases with more than one of the 12 probes tested.

engraftment. The minimum proportion of recipient cells that could have been detected is approximately 5% in this assay, as seen in the control samples in adjacent lanes. The conclusion that stem cell ablation was complete and donor-cell engraftment was successful is particularly strengthened by this genotypic assay since all the cell populations sampled, both peripheral blood and bone marrow, are free of detectable patient cells.

The opposite result was found in the case of patient #3, the recipient of a partial HLA mismatched marrow transplant. Phenotypic and functional analysis of peripheral blood mononuclear cells in this patient suggested that immunologic rejection of donor cells had occurred. This conclusion was supported by the RFLP analysis with LAM4-1214 (Fig 4B) which showed that all (>90%) of the nucleated cells recovered from peripheral blood drawn at 14 days after transplant and cultured in vitro were of host genotype.

One example of lympho-hematopoietic chimerism is shown in Fig 5A. Patient #2 is a stable transplant recipient whose chimeric condition was detected by red cell antigen typing and karyotype analysis. Retrospective analysis of peripheral blood DNA with RFLP probe LAM4-427 demonstrates the progressive repopulation with cells of recipient origin. Peripheral blood cells were exclusively of donor type until 4 months after transplantation, when cells of recipient genotype were first detected at the 10% level. In subsequent months, the proportion of host cells in peripheral blood and in bone marrow has increased to approximately 50%. Despite partial reconstitution with recipient cells, there has been no evidence of recurrence of leukemic cells.

Another example of patient/donor chimerism is shown in Fig 5B (patient #9). By RFLP analysis with probe LAM4-123, exclusive engraftment with donor cells was seen 2 months after transplant. At 5 months posttransplant, peripheral blood cells were 20% of patient origin by RFLP analysis. Leukemic relapse was clinically evident 6 months after transplant, and by this time, the proportion of patient cells as measured by RFLP analysis had risen to 75% in both peripheral blood and bone marrow.

DISCUSSION

Determination of the origin of cells in the lympho-hematopoietic system will be useful for interpreting the course of

Table 2. DNA from 10 Patient-Donor Pairs Was Tested by Hybridization With Each of the 12 Probes Listed in Table 1 and Scored for the Presence of Patient-Specific (P) and Donor-Specific (D) Bands

Probe	Patient/Donor Pair										Number of Cases Informative
	1	2	3	4	5	6	7	8	9	10	
LAM4-427	—	**PD**	—	PD	—	PD	PD	PD	D	D	7
LAM4-1214	**PD**	P	**PD**	PD	—	**PD**	**PD**	D	—	**PD**	8
LAM4-355	PD	D	P	D	—	PD	PD	—	—	—	6
LAM4-159	PD	P	PD	—	PD	—	—	—	—	—	4
LAM4-966	—	PD	D	P	PD	P	PD	—	PD	—	7
LAM4-123	D	PD	PD	—	PD	PD	P	—	**PD**	D	7
LAM4-744	—	—	P	P	PD	PD	P	—	PD	P	7
LAM4-1020	—	PD	D	—	PD	—	D	—	D	P	6
LAM4-1209	—	—	PD	—	PD	—	—	—	PD	—	3
LAM4-1065	—	—	PD	**PD**	**PD**	D	P	**PD**	—	P	7
LAM4-171	—	—	P	—	P	—	—	P	—	D	4
LAM4-962	D	D	—	P	P	—	—	PD	PD	—	6
Total	5	7	9	6	9	7	7	5	5	7	

PD, both patient and donor-informative bands observed in the same DNA hybridization; —, no informative bands. Symbols in boldface indicate the DNA probe chosen for posttransplant analysis of each case.

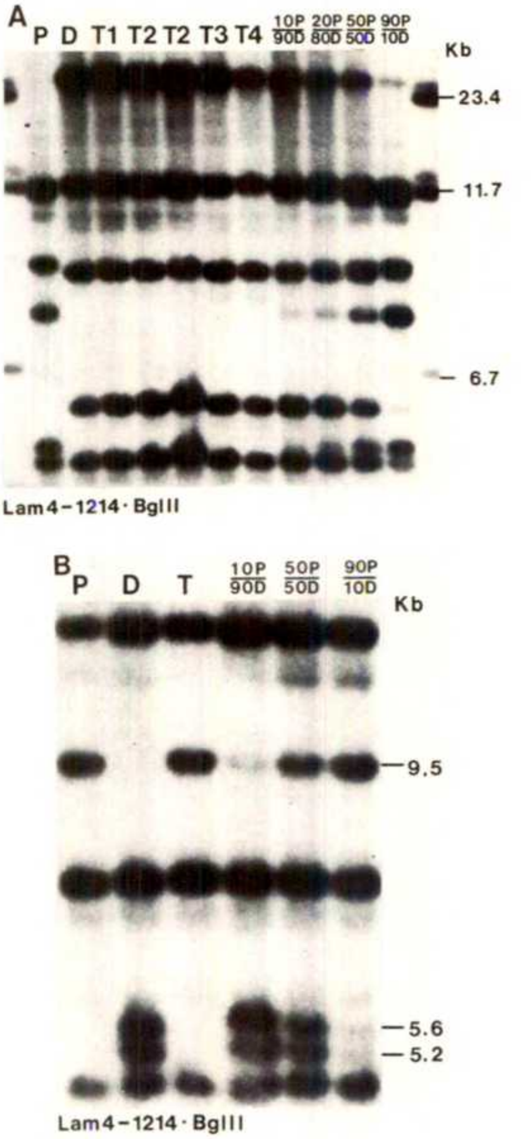

Fig 4. Monitoring engraftment with patient and donor-specific restriction fragments. (A) Successful engraftment in patient #1 is demonstrated in DNA hybridization analysis with probe Lam4-1214 by the persistence of donor-specific fragments (25 kb, 9.3 kb, and 6.0 kb) and the absence of the patient-specific fragment (7.5 kb and 5.6 kb) up to 20 months following transplantation. Faint bands at 10.5 kb and 15 to 20 kb reflect cross-hybridization of the probe with other loci and are not used in diagnosis. P and D indicate pretransplant patient and donor DNA's. T1 to T4 are posttransplant patient DNA samples: T1, peripheral blood (PB) 16 months post-BMT; T2 (2 lanes), PHA-stimulated cultured PB and anti-T12–treated[28] cultured PB, both 17 months post-BMT; T3, bone marrow aspirate, 17 months post-BMT; T4, PB 20 months post-BMT. (B) Failure of engraftment in patient #3. Lane T contains DNA from cultured peripheral blood cells drawn 14 days after transplant. Admixtures of DNA from donor and pretransplant patient blood samples are included as internal standards; eg, 10P/90D indicates 10% patient DNA and 90% donor DNA in the sample. All lanes contain 4 μg of DNA.

events following bone marrow transplantation. The use of DNA polymorphisms for this purpose has been demonstrated to be an informative and sensitive method in several applications. For instance, patient-specific RFLP patterns have been used to confirm the host origin of recurrent leukemic cells after transplantation,[6] and in rare cases, to identify tumors of donor origin.[4,7] Ginsburg et al[5] have detected lympho-hematopoietic chimerism in transplant patients treated for aplastic anemia, SCIDS, and leukemia, and demonstrated different proportions of patient and donor cells in different cell types of a single individual. The results so far have shown the power of RFLP analysis to assay cell populations for the fractions of patient and donor cells with high sensitivity, and also to test cell populations inaccessible with more conventional assays. The results we present here provide further demonstration of the utility of genotypic

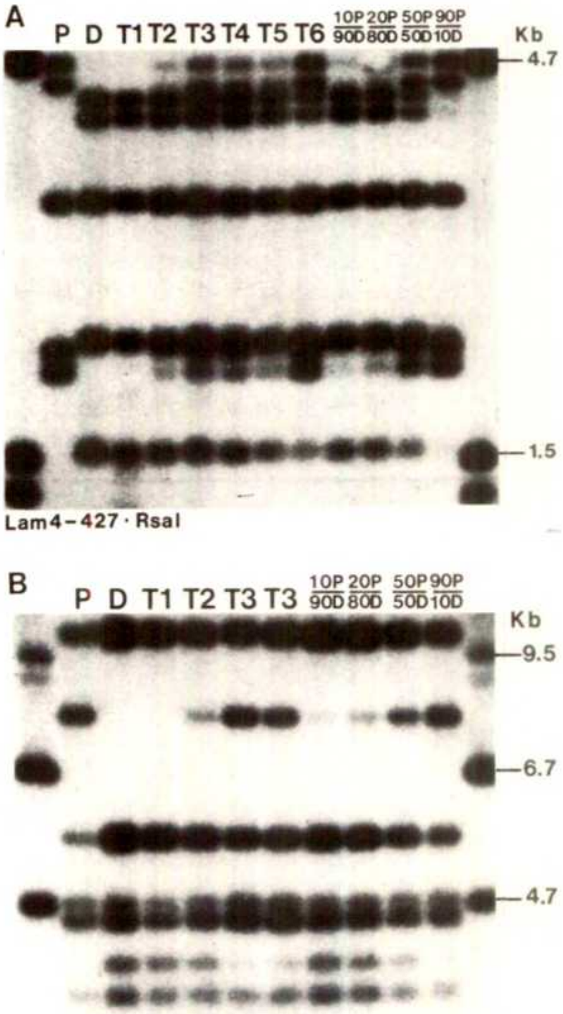

Fig 5. Lympho-hematopoietic chimerism. (A) Engraftment of donor marrow in patient #2 is followed by reappearance of cells of patient origin. P and D, pretransplant patient and donor DNAs, respectively; T1, cultured PB, 3 months post-BMT; T2 to T5, PB at 4, 7, 9, and 12 months post-BMT; T6, bone marrow at 12 months post-BMT. (B) Reappearance of cells of recipient origin in patient #9 after engraftment of donor cells. T1, cultured PB drawn 2 months post-BMT; T2, PB at 5 months post-BMT; T3, PB (left) and bone marrow (right) at 6 months post-BMT. All lanes contain 4 μg of DNA. Proportions of cells of patient and donor genotype in samples showing mixed chimerism are estimated by comparison with mixtures of known composition in adjacent lanes.

analysis to detect mixed chimerism in peripheral blood. In addition, we have applied the test to cells in bone marrow aspirates and found that the proportion of patient and donor cells in peripheral blood reflects the composition of bone marrow cells in several cases. This capability of analyzing any nucleated cell population makes the RFLP test a versatile research tool.

We have increased the usefulness of DNA genotype testing by collecting a set of highly polymorphic probes and making the routine testing of virtually all bone marrow transplant patients feasible. The limitation to RFLP analysis with probes currently used has been the uncertainty of finding adequate patient and donor-specific alleles with any given probe. Because screening probes against pretransplant samples of recipient and donor DNA to find distinguishing bands is the most laborious part of the procedure, probes likely to reveal different alleles are required to make the search practical. Furthermore, because siblings frequently coinherit the same alleles, more than one highly polymorphic probe must be available to screen for informative alleles. Even the highly polymorphic probe, pAW101,[20] with more than eight possible alleles is expected to be uninformative in one third of cases with sibling donors (see Table 1). However, if several highly polymorphic probes are tested, informative markers can be found for virtually all patient-donor pairs except identical twins. We have described here the application of 12 highly polymorphic probes with PIC values ranging from 0.7 to 0.95. Each of the 12 probes was found to be informative in at least three and as many as eight of the ten transplant cases examined, a success rate consistent with the number and frequency of alleles at each of the loci. The net result is that detection of both recipient and donor cells was possible in each case with any of several probes available. We are therefore confident of our ability to find informative alleles for all patients and allogeneic donors. Routine analysis employing only a subset of these probes would be practical and informative in almost all cases.

The advantages of highly polymorphic probes for bone marrow transplant analysis go beyond the probability of discovering informative alleles. Another consequence of the extraordinary variability of these genetic markers is that both patient and donor-specific bands are often displayed with a single probe in a single DNA restriction digest. In this situation, not only is the procedure simplified, but quantitative estimates of proportions of the two cell types are far more reliable. The patient and donor-specific band intensities can be compared directly in the same hybridized sample, without introducing errors due to experimental variation in DNA concentration, gel transfer, or probe hybridization. Of those probes informative for both genotypes, it is generally possible to choose one that displays hybridization patterns suitable for resolution and detection of trace amounts of patient or donor DNA in a mixture of the two. In the optimal pattern, the patient and donor-specific bands are strongly hybridizing and well-resolved by agarose gel electrophoresis. One drawback to the commonly used probe pAW101 is that the allelic EcoRI fragments that must be distinguished are as long as 25 kilobases, at the limit of resolution of agarose gels.

We can also choose several probes to facilitate the routine application of the test by reducing the number of different restriction enzyme digests and gel transfers. As can be seen in Table 1, the polymorphic alleles of the majority of these probes are displayed with only a few different restriction enzymes. By digesting pretransplant samples of recipient and donor DNA with only two enzymes, RsaI and BglII, we can test up to seven of our most highly polymorphic probes and be confident of identifying a probe useful for diagnosis. If for any reason the patient DNA sample is limited in quantity (eg, from an infant or leukopenic patient), the same samples digested with RsaI or BglII could be tested against all seven of these probes by multiple uses of the hybridization filter. Performance of the test requires less than 10μg of DNA per sample, which can generally be obtained from 1 mL of blood.

Examination of the data we have obtained by RFLP analysis of posttransplant samples supports the contention that the test is more sensitive and more reliable than other available methods. In each case in which mixed chimerism developed, the reappearance of patient cells was detected through patient-specific alleles in DNA samples taken at least as early as the first signs of chimerism by karyotype analysis or red blood cell antigens, and in some instances, in advance of other indications. Furthermore, the relative hybridization signals of patient and donor bands give a quantitative measurement of the proportions of the two genotypes. Progressive shifts in the proportion of patient and donor cells were clearly demonstrated in the DNA hybridizations.

We expect that the routine performance of genetic typing of lymphoid and hematopoietic cells will contribute substantially to our understanding of cellular interactions following bone marrow transplantation. In particular, the determination of host or donor origin of cell populations should be pertinent to evaluating graft rejection, graft-versus-host disease, and recurrence of leukemia. The test can also provide the clinician an accurate measure of the completeness of donor engraftment. We have shown that by using a set of highly polymorphic probes we can efficiently perform the diagnosis in virtually all allogeneic transplant cases, so that routine application of the test is feasible.

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The Relation of Platelet Density to Platelet Age: Survival of Low- and High-Density ^{111}Indium-Labeled Platelets in Baboons

By Brian Savage, Paul R. McFadden, Stephen R. Hanson, and Laurence A. Harker

The relationship between platelet density and platelet age has been studied using continuous linear Percoll density gradients and ^{111}In-labeling of autologous platelets in baboons. To investigate changes in platelet density during senescence in the circulation, baboons were infused with ^{111}In-labeled autologous platelets, and blood was collected at one hour postinfusion and twice daily thereafter for six days. Platelets were isolated from these samples in high yield (>95%) and separated in continuous linear Percoll density gradients following density equilibrium centrifugation. Although at one hour postinfusion the density distribution of radiolabeled platelets coincided closely with the distribution of the total platelet population, a detectable symmetrical shift toward higher densities was observed after five days. The relative specific radioactivity (RSR) of high-density platelets (1.064 to 1.067 g/mL) decreased at a slower rate than that of the total platelet population (platelets of all densities), whereas the RSR of low-density platelets (1.053 to 1.056 g/mL) showed a more immediate and rapid decrease. These results give rise to one of two interpretations: (1) low-density platelets have a shorter survival time than more dense platelets and are therefore cleared from the circulation at a faster rate, or (2) platelets of all densities increase in density upon aging in the circulation. To determine the explanation for changing RSR of different density fractions we studied the in vivo disappearance characteristics of low- and high-density ^{111}In-labeled platelets. There were no significant differences between the mean survival times of low-density platelets (5.0 ± 0.49 days, ±1 SD, n = 6), high-density platelets (4.9 ± 0.56 days, n = 6), or control platelets representing platelets of all densities (4.9 ± 0.38 days, n = 6). Although a slight increase in the density of all platelets during platelet senescence is indicated by these studies, we conclude that platelet density heterogeneity is not primarily a consequence of age-related changes in platelet density.

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ALTHOUGH it is well established that platelets are heterogeneous with respect to size, buoyant density, functional capacity, organelle content, and metabolic properties,[1-10] the origin of platelet heterogeneity has been the subject of much controversy. In particular, the widely accepted concept that platelet density heterogeneity may be a consequence, at least in part, of age-related changes in platelet density has recently been challenged. Although several groups of investigators have proposed that platelet maturation in the peripheral circulation is associated with a concomitant decrease in platelet density,[11-15] other groups have concluded either that platelet density increases with platelet age[16-18] or that platelet density heterogeneity is determined during megakaryocyte production, with platelets of different density being derived from megakaryocytes of different ploidy.[7] Therefore, the significance of platelet density heterogeneity to platelet physiology and the clinical relevance of platelet density determinations remain unclear at the present time.

The explanation for the variation in data from different laboratories is not readily apparent, although differences in methodology and the selection of different animal models could account for the discrepant viewpoints. The choice of a suitable density gradient medium and the type of density gradient used are also important considerations. Furthermore, the separation of platelets must be solely on the basis of density without effects of differing platelet sizes.[19] The purpose of the present study was to examine the relationship between platelet density and platelet age in baboons using continuous linear Percoll density gradients under conditions in which true isopycnic density separation of platelets is achieved. We have investigated changes in the density distribution of ^{111}In-labeled autologous baboon platelets following maturation in the peripheral circulation. By studying the in vivo disappearance characteristics of ^{111}In-labeled platelet density cohorts, we have demonstrated that, although a slight increase in the density of all platelets occurs during platelet senescence, platelet survival in baboons is predominantly independent of platelet density.

MATERIALS AND METHODS

Animals studied. Twenty-four juvenile male baboons (*Papio cynocephalus/anubis*) weighing 8 to 14 kg were studied. All animals were dewormed and observed to be disease free for at least 6 weeks prior to use. Circulating platelet concentrations averaged 380,000 ± 91,000/μL (±1 SD) (Variances about the mean are reported as ±1 SD throughout the text.), and white cell counts averaged 8,300 ± 4,100/μL. All animals had a chronic arteriovenous shunt surgically implanted between the femoral artery and vein.[20] The permanent shunt system consisted of two 25-cm lengths of Silastic tubing, 3.0-mm inside diameter (ID) (Dow Corning Corp, Midland, Mich) connected to 13- to 15-gauge Teflon vessel tips (Lifemed, Vernitron Corp, Compton, Calif). In addition, the two Silastic lengths were fixed with Dacron sewing cuffs (Dupont, E.I. de Nemours and Co, Inc, Wilmington, Del) at skin exit sites. The cannulae were sterilized by autoclaving before surgical placement. Blood flow was established by connecting the two Silastic shunt segments with a 1-cm length of blunt-edge Teflon tubing (2.8 mm ID). The permanent Teflon-Silastic shunt did not detectably shorten platelet survival, modify platelet density, or produce measurable platelet activation.[21] For example, plasma levels of platelet factor 4 (PF4), β-thromboglobulin (βTG), and thrombospondin (TSP), and serum thromboxane B_2 (TxB_2) levels were not significantly increased in six animals after permanent shunt placement (Table 1).

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Table 1. Effect of Chronic AV Cannulae on Platelet Activation

	N	Pre	Post	Significance
Plasma PF4 (ng/mL)	6	2.8 ± 1.5	4.6 ± 1.5	$P = .05$
Plasma βTG (ng/mL)	6	3.0 ± 1.3	3.8 ± 1.7	$P > .05$
Plasma TSP (ng/mL)	6	4.3 ± 1.2	5.3 ± 1.8	$P > .2$
Serum TxB_2 (pmol/mL)	6	2,581 ± 903	2,473 ± 1,032	$P > .5$
Mean platelet density (g/mL)	5	1.062 ± 0.004	1.062 ± 0.003	$P > .5$
Platelet ADP (μmol/10^{11} platelets)	5	4.86 ± 2.27	4.73 ± 2.17	$P > .5$
Mean platelet volume (fL)	6	7.05 ± 0.63	7.13 ± 0.56	$P > .5$

Abbreviations: AV, arteriovenous; ADP, adenosine diphosphate.

All values are expressed as mean ± SD.

Density gradients. Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) was mixed with an equal volume of buffered saline-glucose-citrate (BSGC) consisting of KH_2PO_4 (0.218 g/L), Na_2HPO_4(1.22 g/L), NaCl (6.83 g/L), $Na_3C_6H_5O_7 \cdot 2H_2O$ (4.00 g/L), glucose (2.00 g/L), prostaglandin E_1 (PGE_1) (0.0004 g/L), pH 7.4, osmolarity 295 mosm/kg. Continuous sigmoidal density gradients were prepared by centrifuging the mixture (25 mL) for 20 minutes at 25,400 g in 50-mL polycarbonate tubes in a fixed-angle rotor (Sorvall, type SS-34, Ivan Sorvall, Inc, Newtown, Conn). These gradients were used for the isolation of platelets from whole blood (see the following). Linear Percoll density gradients, used for the density separation of platelets, were prepared in the following manner. Percoll was made iso-osmotic with physiologic BSGC solution by adding nine parts (vol/vol) of Percoll to one part of 10× concentrated BSGC buffer, pH 7.4. The osmolarity of the resulting stock solution was adjusted to 295 mosm/kg H_2O with deionized water to give a stock isotonic Percoll (SIP) solution. Continuous linear density gradients (30 mL) ranging in density from 1.045 to 1.110 g/mL were prepared in 50-mL polycarbonate tubes using two-chamber gradient maker (Buchler Instruments, Fort Lee, NJ).

Platelet isolation. The isolation of platelets of all densities from whole blood was achieved by a modification of the procedure described by Martin et al.[22] Acid-citrate-dextrose (ACD) anticoagulated whole blood (5 mL) was diluted with an equal volume of BSGC, layered onto a sigmoidal, self-generated Percoll density gradient, and centrifuged at 22 °C for ten minutes at 400 g in 50-mL polycarbonate tubes using a horizontal swing-out rotor (International Equipment Co, Needham Heights, Mass). Under these conditions, cells are separated primarily on the basis of size.[22] Platelets were harvested from these gradients in high yield (mean recovery, 93% ± 3%; leukocyte contamination <0.1%) using a wide-bore plastic pipette.

Platelet density separation. Platelet preparations (up to 5.0 × 10^7 platelets per gradient) were layered by hand using a plastic pipette onto linear Percoll density gradients and centrifuged at 2,250 g for 90 minutes at 22 °C using a horizontal swing-out rotor. In pilot studies, time-dependent modifications in the platelet distribution in the gradient were studied to determine the optimum centrifugation time required for platelets to reach their equilibrium position in the density gradient. True isopycnic separation of platelets was achieved using the aforementioned conditions. Gradients were fractionated by upward displacement with undiluted iso-osmotic Percoll by using a narrow probe (0.5 mm ID) inserted from the top of the gradient tube, and 40 fractions were collected using an automatic fraction collector. The linearity of density gradients was monitored by measuring the refractive index of fractions collected from control gradients (containing no platelets). Colored density marker beads (Pharmacia) were used to calibrate density gradients.

Platelet labeling. Autologous baboon platelets were labeled in vitro with ^{111}In-oxine in the following manner. Whole blood (100 mL) was collected directly into plastic bags (TA-3, Fenwal Labs, Deerfield, Ill) containing 20 mL ACD anticoagulant (National Institutes of Health formula A). The blood was centrifuged in the bag at 300 g for ten minutes. The supernatant platelet-rich plasma (PRP) was transferred to a second bag and the pH adjusted to 6.5 by the addition of 0.15 mol/L citric acid (0.1 mL/10 mL PRP). The platelets harvested in this way (yield, approximately 70%) were representative of the whole platelet population in terms of mean platelet volume, mean platelet density, and dense-granule adenine nucleotide content (this laboratory, unpublished observations). The RBC fraction was returned to the donor animal. The PRP was centrifuged at 1,300 g for 15 minutes to form a platelet pellet. The supernatant platelet-poor plasma (PPP) was removed and the platelet pellet washed once by overlaying with 30 mL of Ringer's citrate dextrose (RCD, pH 6.5) to remove residual plasma proteins. The pellet was then gently resuspended in 5.0 mL RCD and incubated for 30 minutes with 500 to 1,000 μCi ^{111}In-oxine (Amersham Corp, Arlington Heights, Ill). Contaminating red cells were removed by a final slow centrifugation at 200 g for five minutes. Labeling efficiency was determined by diluting 200 μL of the labeled platelet concentration with 5.0 mL RCD and comparing the activity in 0.5 mL of the diluted platelet suspension with the activity in 0.5 mL of cell-free supernatant following centrifugation at 3,000 g for 30 minutes. In the present study, labeling efficiency was 89% ± 7%.

Survival of low- and high-density ^{111}In-labeled platelets. Low- and high-density ^{111}In-labeled platelet cohorts were prepared by separating total-population ^{111}In-labeled platelets on linear Percoll density gradients as previously described. Platelet number and platelet-associated radioactivity were measured in all fractionated samples. Platelets with densities in the range 1.052 to 1.056 g/mL (representing approximately 17% of the total number of platelets) were combined to give a labeled low-density platelet subpopulation. Similarly, platelets with densities in the range 1.064 to 1.068 g/mL (representing approximately 17% of the total number of platelets) were combined to yield high-density labeled platelets. Baboons were infused with either low- or high-density autologous labeled platelets (10 mL, containing approximately 10^8 platelets). Blood (4 mL) was collected in 2 mg/mL EDTA at 90 minutes postinfusion and twice daily thereafter for six days. In control studies, platelet density fractions were recombined, and comparable amounts of autologous, labeled platelets (representing platelets of all densities) were infused into baboons. Pilot studies showed that infusion of Percoll alone did not have a measurable effect on the hematocrit value, platelet count, or the survival of circulating platelets. Platelet ^{111}In activity, hematocrit values, and platelet counts were determined on all serial blood samples. Platelet-associated ^{111}In activity was determined by measuring whole blood ^{111}In activity (1.0 mL) and correcting for any residual plasma ^{111}In activity remaining from previous experiments. Plasma activity per milliliter of whole blood was calculated by multiplying the activity per milliliter of platelet-free plasma by the factor 1 - hematocrit. A transient sequestration of labeled platelets was observed when the recovery of ^{111}In-labeled platelets was calculated in samples measured at 30, 60, 90, and 120 minutes following infusion of the labeled platelet preparation; typical values were 70%, 78%, 88%, and 89% respectively. After 90 minutes, no further increases in the circulating platelet radioactivity were seen. Mean platelet survival times were calculated by fitting the disappearance curve of platelet radioactivity to γ-functions as described by Murphy et al.[23,24] The intercept on the y axis (^{111}In platelet activity) at $t = 0$ was assigned the value of 100%; all subsequent time points were expressed as a percentage of this value. Thus, data points from individual experiments could be effectively combined in a single figure. The proportion of labeled platelets remaining within the systemic circulation (recovery) was calculated from the initial platelet activity per milliliter of whole blood multiplied by the estimated blood volume (65 mL/kg) and divided by the ^{111}In activity injected.

Age-related changes in the density distribution of total-population labeled platelets. In these studies, baboons were transfused with ^{111}In-labeled autologous platelets (representing platelets of all densities). Blood was sampled at one hour following infusion of labeled platelets and at 24-hour intervals for six days. Platelets were isolated from whole blood and separated on linear Percoll density gradients as previously described. Platelet number and ^{111}In activity were measured in each density fraction. The specific radioactivity of each fraction was determined by dividing the platelet number by the platelet-associated radioactivity. The relative specific radioactivity (RSR) at time *t* following infusion of labeled platelets is defined as the specific activity at time *t* divided by the initial specific activity at one hour postinfusion.

To determine whether prior exposure to Percoll of ^{111}In-labeled platelets alters their in vivo aging behavior, baboons were transfused with ^{111}In-labeled autologous platelets (representing platelets of all densities) that had previously been exposed to Percoll. In these studies, ^{111}In-labeled platelets were separated on the basis of density on linear Percoll density gradients using the conditions previously described. Following fractionation of gradients, platelet density fractions were combined and a representative fraction infused into baboons (5×10^8 to 10^9 platelets in 15 mL total volume). Blood was sampled (5 mL, ACD anticoagulant) at 90 minutes and at five days following infusion of labeled platelets. Platelets were isolated from whole blood and separated on linear Percoll density gradients as previously described. Platelet number and platelet-associated ^{111}In activity were measured in all density fractions.

Effect of Percoll treatment on platelet function in vivo. Infusion of bovine skin type 1 collagen directly into the circulation of normal baboons produces a partially reversible reduction in circulating platelet concentrations (see Results). This methodology was used to compare quantitative alterations in circulating platelet characteristics resulting from collagen-induced platelet aggregation in vivo. Baboons were infused with either ^{111}In-labeled autologous platelets or ^{111}In-labeled autologous platelets previously exposed to Percoll as previously described. At two hours following infusion of labeled platelets, baboons were injected with bovine skin type 1 collagen (a gift from Collagen Corp, Palo Alto, Calif) at a dose previously shown to cause approximately a 50% reduction in circualating platelet concentrations (4.0 mg/kg in 15 ml of phosphate-buffered saline, pH 7.2). Blood (4.0 mL) was sampled into 2 mg/mL EDTA at 5, 10, 15, 30, 60, 120, and 180 minutes for the determination of platelet count, hematocrit value, and platelet ^{111}In activity. Platelet-associated ^{111}In activity was determined by measuring whole blood ^{111}In activity (1.0 mL) and correcting for plasma ^{111}In activity. Plasma activity per milliliter of whole blood was calculated by multiplying the activity per milliliter of platelet-free plasma by the factor 1- hematocrit. Platelet-specific radioactivity was calculated by dividing the platelet-associated ^{111}In activity per milliliter of whole blood by the platelet number per milliliter of whole blood.

Other methods. Radioactivity was measured using a gamma spectrometer (Nuclear Chicago, Chicago). Concentrations of platelet suspensions were determined with an electronic cell counter (Coulter Model ZBI, Coulter Electronics, Hialeah, Fla). Whole blood platelet counts and platelet volume measurements were performed using a Model 810 platelet analyzer and a Model 106 diluter interfaced with a Model 150 cell counter (Baker Instruments Corp, Allentown, Pa).

Blood (5 mL) for PF4 and βTG was drawn into precooled syringes containing 1 mL of ACD, acetylsalicylic acid, and PGE$_1$, 30 mmol/L and 1 mol/L final concentrations respectively. Assays for PF4 and βTG were performed by competitive radioimmunoassay as described previously,[21] whereas the TSP assay was that described by Dawes et al.[25] The production of thromboxane A$_2$, measured as the stable metabolite of TxB$_2$ in whole blood, was determined using a radioimmunoassay (New England Nuclear, Boston) for samples collected by the method described previously.[26] Platelet adenine nucleotide levels were measured by high-performance liquid chromatography of neutralized perchloric acid extracts.[27]

RESULTS

The frequency distribution of baboon platelet densities in linear Percoll density gradients was predominantly Gaussian (Fig 1). Platelet density ranged from 1.049 to 1.075 g/mL, with a mean platelet density of 1.061 g/mL. The platelet distribution in the gradient remained constant on increasing the centrifugation time, indicating that platelets had reached their density equilibrium position and were not being separated partially on the basis of volume. Platelet recovery was 91% ± 8% (n = 8). When blood was sampled by venipuncture from baboons without shunts, the platelet density distribution was not significantly different from that shown in Fig 1. The size characteristics of density-dependent platelet subpopulations were studied. Baboon platelet density-dependent subpopulations were log-normally distributed in size. The platelet mean cell volume measured in citrated whole blood was 7.18 ± 0.58 fL (n = 8). The corresponding value measured after combining the density-dependent subpopulations was 7.04 ± 0.46 fL (n = 8), a value that was not significantly different from that obtained with the unfractionated whole blood ($P > .1$). Thus, this control study shows that artifactual changes in platelet volume did not occur during the density separation procedure. A direct relationship between platelet density and mean platelet volume was observed, up to a density of 1.060 g/mL, with volume

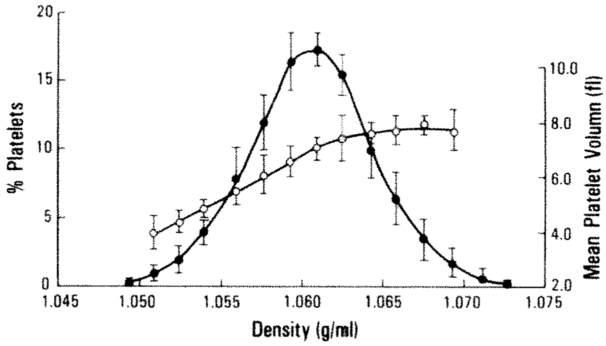

Fig 1. Density and size distribution of baboon platelets. Platelets were separated on linear continuous Percoll density gradients (solid circles); the platelet mean cell volume is shown by the open circles. Error bars represent ±1 SD (n = 8).

plateauing at densities above 1.063 g/mL (Fig 1). To determine the effect of chronic AV cannulae on circulating platelet properties, studies were performed in animals both with and without cannulae. Both groups were equivalent in terms of mean platelet density, mean platelet volume, plasma α-granule proteins, platelet ADP, and platelet capacity to synthesize thromboxane A_2 (Table 1). Normal platelet survival in baboons with chronic AV cannulae has previously been documented from this laboratory.[21,28]

At one hour following infusion of ^{111}In-labeled autologous baboon platelets, the density distribution of ^{111}In-labeled platelets coincided closely with the distribution of total platelets, indicating that the labeling procedure did not affect platelet density and that platelets of all densities were labeled in an equivalent manner (Fig 2A). The platelet density distribution profile remained constant for a period of five days, as would be expected under basal physiologic conditions (Fig 2B). However, there was a detectable transition in the distribution of ^{111}In-labeled platelets toward platelets of higher densities (the peak density shifted from 1.061 to 1.063 g/mL; $P < .001$, X^2 test). This shift was most apparent by day 5 when the labeled platelets remaining in the circulation represented a relatively old platelet population. The average platelet life span in baboons is 5.5 ± 0.4 days.[20,28] Essentially identical results to those shown in Fig 2 were obtained when ^{111}In-labeled platelets previously exposed to Percoll were used (Fig 3), indicating that prior exposure of labeled platelets to Percoll or to the centrifugation conditions used did not affect their in vivo aging properties with respect to density.

When the specific radioactivity of low- and high-density platelet subpopulations (1.053 to 1.056 g/mL and 1.064 to 1.067 g/mL, respectively) was expressed as a percentage of the corresponding initial values (at one hour postinfusion), the results shown in Fig 4 were obtained. The RSR of the total platelet population (platelets of all densities) decreased in a linear manner typical of the disappearance pattern of ^{111}In-labeled platelets in normal baboons.[28] In contrast, the RSR of high-density platelets remained constant during the first 42 hours and decreased relatively slowly thereafter, whereas the RSR of low-density platelets showed a more immediate and rapid decrease (Fig 4).

The differential loss of radioactivity from low-density platelets in concert with a relative enrichment in the radioactivity of more dense platelets is compatible with either of the following interpretations: (1) platelets of all densities increase in density on aging in the circulation, producing a shift in the total platelet population toward higher densities; or (2) low-density platelets have a shorter survival time and are therefore cleared from the circulation at a faster rate than more dense platelets. To distinguish between these two possibilities, the survival characteristics of low-density (1.052 to 0.056 g/mL) and high-density (1.064 to 1.068 g/mL) ^{111}In-labeled platelets were investigated. When ^{111}In-labeled low-density platelets were infused into baboons, measurements of the platelet density distribution of circulating platelets revealed that labeled platelets were primarily restricted to the low-density region at 90 minutes postinfusion (Fig 5A), whereas following infusion of ^{111}In-labeled high-density platelets, labeled platelets were located in the high-density region (Fig 5B). The recoveries of ^{111}In-labeled platelets at two hours postinfusion were 94.3% ± 6.4% and 89.8% ± 7.1% for low- and high-density platelets respectively.

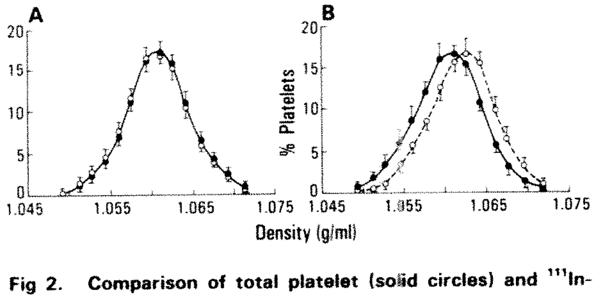

Fig 2. Comparison of total platelet (solid circles) and ^{111}In-labeled platelet (open circles) density distributions. Platelets were separated in linear continuous Percoll density gradients at one hour (A) and at five days (B) following infusion of ^{111}In-labeled autologous platelets in baboons. Error bars represent ±1 SD (n = 6).

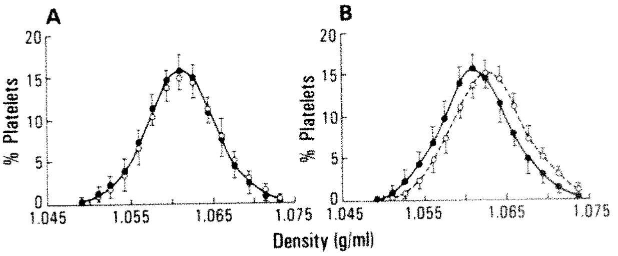

Fig 3. Effect of Percoll on total platelet (solid circles) and ^{111}In-labeled platelet (open circles) density distributions. Platelets were separated in linear continuous Percoll density gradients at one hour (A) and at five days (B) following infusion of ^{111}In-labeled autologous baboon platelets that had previously been exposed to Percoll for two hours in vitro.

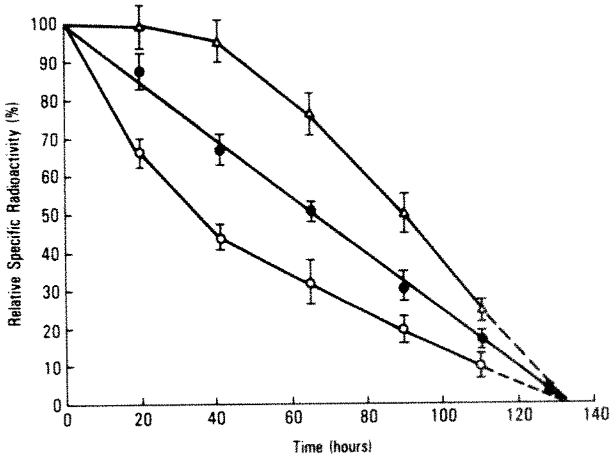

Fig 4. Comparison of relative specific platelet radioactivity of density cohorts. High-density (triangles), low-density (open circles), and total-population (solid circles) platelets following infusion of ^{111}In-labeled autologous platelets in normal baboons are shown. The specific radioactivity is expressed as a percentage of the initial specific activity (at one hour postinfusion) for each density subpopulation. Error bars represent ±1 SD (n = 6).

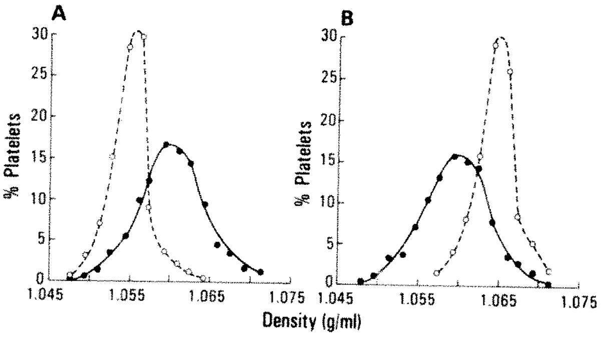

Fig 5. Platelet density distributions. Distributions are shown for total platelets (solid circles) and ^{111}In-labeled platelets (open circles) at 90 minutes following infusion of autologous ^{111}In-labeled low-density (1.052 to 1.056 g/mL) (A) and high-density (1.064 to 1.068 g/mL) (B) baboon platelets.

There were no significant differences between the mean survival times of low-density platelets (5.0 ± 0.49 days, mean ± SD; n = 6), high-density platelets (4.9 ± 0.56 days, mean ± SD; n = 6) or control platelets representing platelets of all densities (4.9 ± 0.38 days, mean ± SD; n = 6) (Fig 6). The mean platelet survival time of control platelets was normal and equivalent to results previously reported for baboons using both ^{51}Cr- and ^{111}In-labeled platelets.[28]

To determine whether labeled platelets previously exposed to Percoll were functionally normal in vivo, relative reactivity to infused collagen was assessed. Infusion of bovine skin type 1 collagen (4.0 mg/kg) into normal baboons resulted in a partially reversible reduction in the circulating platelet count (Table 2). Maximal reductions in platelet numbers occurred at approximately five minutes following collagen injection (54% ± 6% of initial value, n = 4). After three hours, 26% ± 4% of the sequestered platelets had returned to the circulation. The results of collagen infusion studies in baboons previously infused with ^{111}In-labeled platelets or ^{111}In-labeled, Percoll-treated platelets are summarized in Table 2. In both cases, a 42% to 45% reduction in the circulating platelet concentration was observed that was partially reversible after 180 minutes. Furthermore, the platelet specific radioactivity (expressed as platelet-associated cpm/platelet number) remained constant throughout, indicating that both labeled platelets and labeled platelets previously exposed to Percoll were equally reactive toward collagen in vivo compared with the unlabeled circulating platelet population. No significant changes in mean platelet volume were observed following collagen infusion (Table 2).

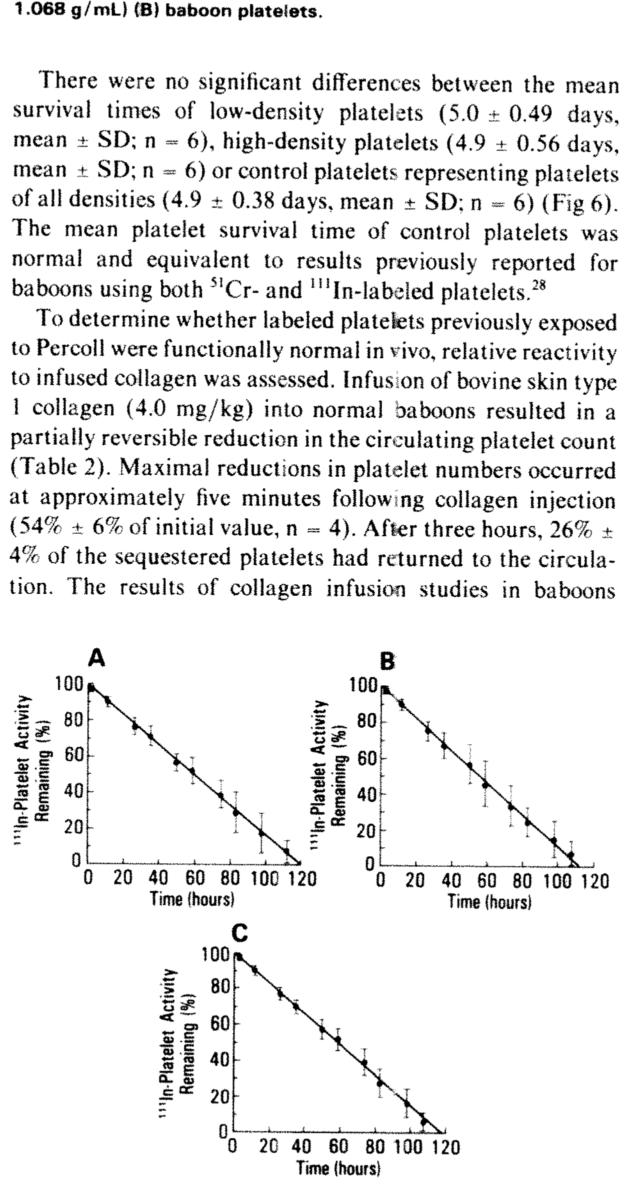

Fig 6. In vivo ^{111}In platelet disappearance patterns. ^{111}In-labeled low-density platelets (A), high-density platelets (B), and control platelets representing platelets of all densities (C) are compared. Error bars represent ±1 SD (n = 6).

DISCUSSION

Although these studies show a slight increase in the density of platelets during platelet senescence, the principal finding is that platelet preparations with different mean densities have similar survival times.

The pattern of survival of normal control baboon (and human) platelets is essentially linear,[29-31] indicating that platelet survival is predominantly determined by senescence in the circulation and not by random destruction or ongoing platelet use in the maintenance of basal hemostasis. Since the separation of platelets on the basis of density does not provide an adequate means of isolating or identifying platelet age cohorts in baboons, the reported clinical usefulness of measuring platelet density as a means of estimating platelet turnover in humans[32] must be questioned,[16-18] although in clinical conditions associated with an increased platelet turnover such as acute thrombosis an increase in low-density platelets has been reported.[33]

The long-standing controversy concerning the origin of platelet density heterogeneity probably reflects, in part, the different methodologies used for the density separation of platelets. Previous studies have used sucrose,[34] inert silicone oils,[1,2] albumin,[18] arabino-galactan polysaccharide (Stractan),[14,15,17] and Percoll[35] gradient systems. Such studies reported by several different groups have produced conflicting data, leaving unclear whether platelet aging in the circulation is associated with (1) an increase in platelet density,[16-18] (2) a decrease in platelet density,[11-15] or (3) no change in platelet density.[36-38] The physical characteristics of the gradient medium often impose important limitations on the use of density gradient centrifugation systems. Furthermore, theoretical considerations suggest that major constraints are imposed by discontinuous gradient techniques[19] since platelets of variable density may be trapped at multiple-step interfaces resulting in stress-induced platelet activation. Since the main variables that determine the separation of platelets in density gradients are platelet volume and density, the viscosity of the medium, and the speed of centrifugation,[19] the use of high-viscosity gradients (eg, Stractan) will necessitate longer centrifugation times if true platelet density separation is to be achieved. If centrifugation is stopped before equilibrium conditions have been reached, platelet volume will affect the distribution. In view of these considerations, it is now apparent that the true density distribution of platelets has probably not been measured in many previous studies. Thus, it seems likely that properties

Table 2. Failure of Percoll to Affect Platelet Reactivity to Collagen In Vivo

Time (min)	Platelet Count ($\times 10^3/\mu L$)		Platelet Specific Radioactivity (cpm/Platelet Count)		Mean Platelet Volume (fL)	
	Untreated	Percoll-treated	Untreated	Percoll-treated	Untreated	Percoll-treated
0	508.5 ± 46.5	406.6 ± 30.3	1,790.5 ± 50.6	1,785.9 ± 40.3	6.5 ± 0.18	6.8 ± 0.24
5.0	294.9 ± 55.9	228.4 ± 20.6	1,668.0 ± 48.9	1,794.5 ± 63.8	6.4 ± 0.23	6.6 ± 0.19
10.0	298.3 ± 42.9	234.7 ± 23.0	1,647.5 ± 65.3	1,773.4 ± 14.8	6.4 ± 0.25	6.7 ± 0.21
15.0	312.2 ± 50.0	248.9 ± 31.9	1,840.0 ± 51.7	1,775.2 ± 35.1	6.5 ± 0.17	6.6 ± 0.15
30.0	323.5 ± 43.2	270.3 ± 24.0	1,797.0 ± 38.2	1,798.3 ± 32.4	6.3 ± 0.21	6.6 ± 0.19
60.0	361.0 ± 49.2	288.4 ± 47.7	1,737.3 ± 56.8	1,826.5 ± 41.3	6.6 ± 0.20	6.8 ± 0.23
120.0	381.4 ± 48.5	316.7 ± 48.4	1,791.5 ± 45.3	1,799.0 ± 70.4	6.5 ± 0.18	6.7 ± 0.25
180.0	396.6 ± 53.4	323.5 ± 47.3	1,754.2 ± 49.2	1,793.5 ± 52.1	6.5 ± 0.19	6.5 ± 0.18

Collagen (4 mg/kg) was infused via a chronic AV shunt surgically implanted between the femoral artery and vein.
All values are expressed as mean ± SD ($n = 4$).

previously attributed to platelet density subpopulations may, in fact, reflect platelet subpopulations varying in both density and volume.

The selection of an appropriate animal model is also an important consideration. The hemostatic mechanism in the baboon closely resembles that of humans; the pattern of survival of human and baboon platelets is predominantly linear, indicating that platelet survival in both species is primarily affected by senescence in the circulation.[29-31,39,40] In contrast, the rabbit model used in previous studies[13,15] may not be appropriate for simulating conditions in humans since the platelets in this species differ from human platelets in several respects.[41] For example, the ratio of random to constant destruction of platelets in rabbits is threefold higher than in humans;[42] the aging hypothesis of platelet heterogeneity relies on a process that is independent of random platelet destruction. In the present study, the distribution of ^{111}In-labeled baboon platelets in linear continuous Percoll density gradients was investigated using conditions in which platelets are separated entirely on the basis of density (ie, true isopycnic separation). We conclude that the shift in the distribution of ^{111}In-labeled platelets toward higher densities represents a slight increase in the density of platelets during peripheral maturation. Although low-density platelets would be expected to be enriched with younger platelets on the basis of this mechanism, analysis of Fig 2B shows that old platelets (^{111}In-labeled platelets remaining in the circulation at five days postinfusion) display a broad dispersion in density, indicating that narrow-range platelet density cohorts contain platelets of all ages: differences in the mean platelet age of high- and low-density platelet populations could not be detected by their in vivo disappearance characteristics in the present study.

Our data support the concept that platelet density heterogeneity is primarily determined during the thrombocytopoietic process. The slight increase in the density of all platelets occurring during their subsequent life span in the circulation contributes little to the density heterogeneity of platelets. This interpretation is supported by the work reported by Martin and Penington[35] who demonstrated a 5% increase in the mean density of platelets in monkeys five days after infusion of ^{51}Cr-labeled autologous platelets and by Boneu and co-workers[34] in human subjects following autologous ^{51}Cr-labeled platelet survival studies. However, our data contrast with the observations reported by Corash and co-workers[14] using discontinuous Stractan gradients and rhesus monkeys and by Rand and co-workers using similar methods and rabbits.[15]

Conflicting data from different laboratories are, in fact, difficult to compare since platelets from different species display considerable variation with respect to structural,[43,44] kinetic,[36] functional,[45] and biochemical[39] properties. The problem is further compounded by differences in experimental approach and methodology and different interpretations of similar data. In view of the recent studies by Mezzano and co-workers,[46] it is now apparent that the magnitude and direction of age-related changes in platelet density are, to some extent, dependent on the species studied. In this context, mongrel dog platelets were found to decrease in density with aging,[46] in direct contrast to previous observations with human subjects,[17] even though the experimental approach and methodology in the two species were essentially the same.

Our data show that, in baboons, platelets of all densities increase slightly in density during aging in the circulation. However, because of the broad spectrum and degree of overlap of densities among platelet age cohorts, no significant correlation was found between platelet density and platelet age.

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Coagulation-Cancer Interaction In Situ in Renal Cell Carcinoma

By Leo R. Zacharski, Vincent A. Memoli, and Sandra M. Rousseau

Fibrin was detected by immunospecific techniques associated with both intravascular and extravascular tumor deposits in renal cell carcinoma. In addition, coagulation factors VII and X were demonstrated in intercellular spaces within tumor tissue and adjacent to the surface of tumor cells. Scant accumulation of platelets on intravascular tumor masses was observed. These data suggest that tumor cells in renal cell carcinoma may induce fibrin formation locally by a factor VII–mediated (and thus tissue factor–initiated) pathway of blood coagulation. This mechanism may also account for the hypercoagulable state that exists with this tumor type. We postulate that local fibrin formation may contribute to the growth and spread of this particular type of cancer and that the course of renal cell carcinoma may be ameliorated by anticoagulant therapy.

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NEOPLASTIC CELLS may interact with the coagulation mechanism by initiating the sequence of reactions that leads to fibrin formation[1] or by inducing platelet aggregation.[2] Since treatment of tumor-bearing experimental animals with anticoagulant or antiplatelet drugs reduces metastatic dissemination, it has been proposed that local coagulation activation in vivo at the host-tumor interface may, at least in part, mediate expression of neoplastic behavior by malignant cells. The literature supporting this hypothesis has recently been reviewed.[1,2]

Cause-and-effect relationships between coagulation activation and tumor dissemination could conceivably exist for human as well as experimental animal malignancy. Evidence for activation of the coagulation mechanism can frequently be obtained in cancer patients through appropriate laboratory tests.[3] Unfortunately, the consequences of administering antithrombotic drugs to patients with malignancy remain undefined because carefully controlled clinical trials of anticoagulant and antiplatelet agents have only recently been undertaken.[2,4,5] However, evidence for a beneficial effect of warfarin in small-cell carcinoma of the lung (SCCL)[4,6] together with evidence for variability in warfarin responsiveness between human tumor types, as exists for experimental tumors,[5] provides an incentive for further study.

Information on the mechanisms of tumor cell interaction with the host coagulation mechanism has been sought by attempting to identify the end product of coagulation activation, fibrin, in association with tumor masses. Detailed studies of breast cancer,[7] lymphomas,[8] and SCCL[9] have shown that fibrin is deposited in different patterns associated with viable tumor cells in these particular tumor types. In addition, evidence has been presented for the existence of the putative initiator of coagulation, tissue factor, on SCCL cells themselves.[9]

Although some progress has thus been made in defining the coagulation–cancer cell interaction in human malignancy, information on the occurrence of fibrin in other human tumor types is lacking. Further uncertainties surround the precise mechanism by which fibrinogen is converted to fibrin and the possible role of platelets as well as fibrinolytic enzymes. The purpose of this paper is to describe observations on the occurrence of certain coagulation reaction intermediates and fibrin in renal cell carcinoma (RCC). This particular tumor type was chosen for investigation because it is commonly accompanied by activation of the coagulation mechanism and because of its propensity to flourish in thrombi within vascular channels (see Discussion). Emphasis is placed on establishing methods for detection of coagulation factors VII and X to test for the existence of these coagulation intermediates of the extrinsic pathway in association with tumor cells.

MATERIALS AND METHODS

The avidin-biotin complex (ABC) technique was adopted from the method of Hsu et al[10] using reagents from Vector Laboratories (Burlingame, Calif). Immunofluorescence methods using a specific, polyclonal antibody to human fibrinogen were those described previously for detection of fibrin in SCCL.[9] Immunofluorescence methods using a specific, polyclonal antibody to human fibrinogen were those described previously for detection of fibrin in SCCL.[9] Antibody to human fibrinogen was obtained from Cappel Laboratories (Malvern, Penn). The specificity of this antibody was demonstrated previously.[9] Rabbit antibody to human factor VIII antigen was obtained from Behring Diagnostics (American Hoechst Corp, Somerville, NJ). Monoclonal antibodies to human factors VII and X were obtained from Dr Walter Kisiel,[11] and polyclonal goat antibody to human factor VII was obtained from Dr Paul Bajaj.[12] Mouse monoclonal antibody to the IIb-IIIa surface glycoprotein of human platelets was obtained from Dr Barry Coller.[13] The specificity of these antibodies was demonstrated by these investigators. Dilutions of antibodies used were generally 1:50, 1:100, and 1:200. The same dilutions were used for both control and experimental sections and were chosen for optimal end point.

Tissue was obtained from surgically resected tumor specimens in three previously untreated cases of RCC. Platelet counts, prothrombin times, and activated partial thromboplastin times performed on these three cases were normal. Blocks of fresh tumor tissue from the peripheral regions of the tumor were prepared and stored as described previously.[9] Fresh-frozen tissue sections were studied either unfixed for immunofluorescence[9] or fixed in acetone for the ABC technique.[10] Greater sensitivity was achieved with the immunoperoxidase procedure. However, immunofluorescence was perferred over the immunoperoxidase procedure for demonstration of fibrin because fixation required for the latter procedure reduced reactivity of antifibrinogen antibody with fibrin.[9] In some instances the ABC method was applied to formalin-fixed, paraffin-imbedded RCC tissue maintained in the pathology laboratory. Appropriate control studies included omission of the primary antibody and replacement with nonimmune sera, use of primary antibodies having other specificities, and the use of a positive control tissue for each

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antibody tested. The positive control for the antibody to fibrinogen was term placenta; for antibody to factors VII and X, normal liver; for antibody to factor VIII antigen, vascular endothelium; and for antibody to platelet IIb-IIIa glycoprotein, peripheral blood platelets and bone marrow megakaryocytes.

RESULTS

Dense specific fluorescence was observed in sections of fresh-frozen tissue with antifibrinogen antibody that revealed fibrin encasing RCC tumor cells within thin-walled vascular channels (Fig 1) and in the fibrous connective tissue stroma surrounding viable tumor cells in extravascular sites (Fig 2). Phase-contrast–fluorescence photographic pairs of the same microscopic field demonstrated relationships between tumor cells and fibrin. Individual tumor cells in a tumor mass within vascular channels were encased in dense fibrin-specific fluorescence, but connective tissue was lacking (Fig 1). By contrast, fibrin-containing connective tissue surrounded nests of tumor cells within the tissue (Fig 2). The adjacent normal kidney did not contain fibrin, indicating that the fibrin was specifically related to the tumor itself.

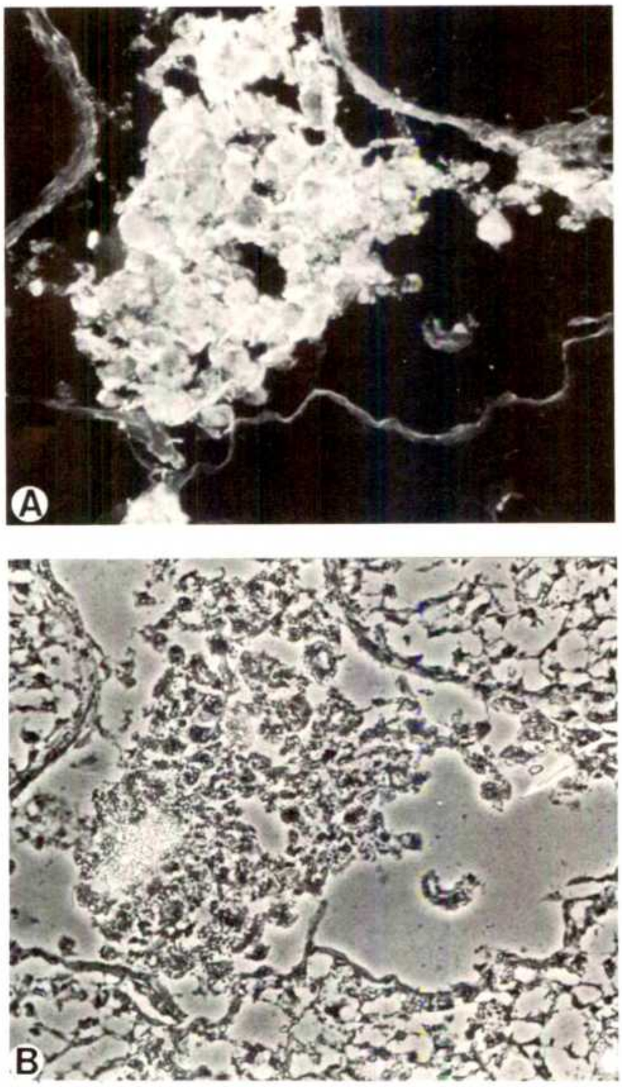

Fig 1. Dense specific fluorescence for fibrin encasing an intravascular tumor mass in a fresh-frozen section of RCC observed by fluorescence (A) and phase-contrast (B) microscopy. Individual tumor cells are evident by phase contrast within the mass of fibrin demonstrated by fluorescence. There is no obvious connective tissue reaction (original magnification × 160; current magnification × 123).

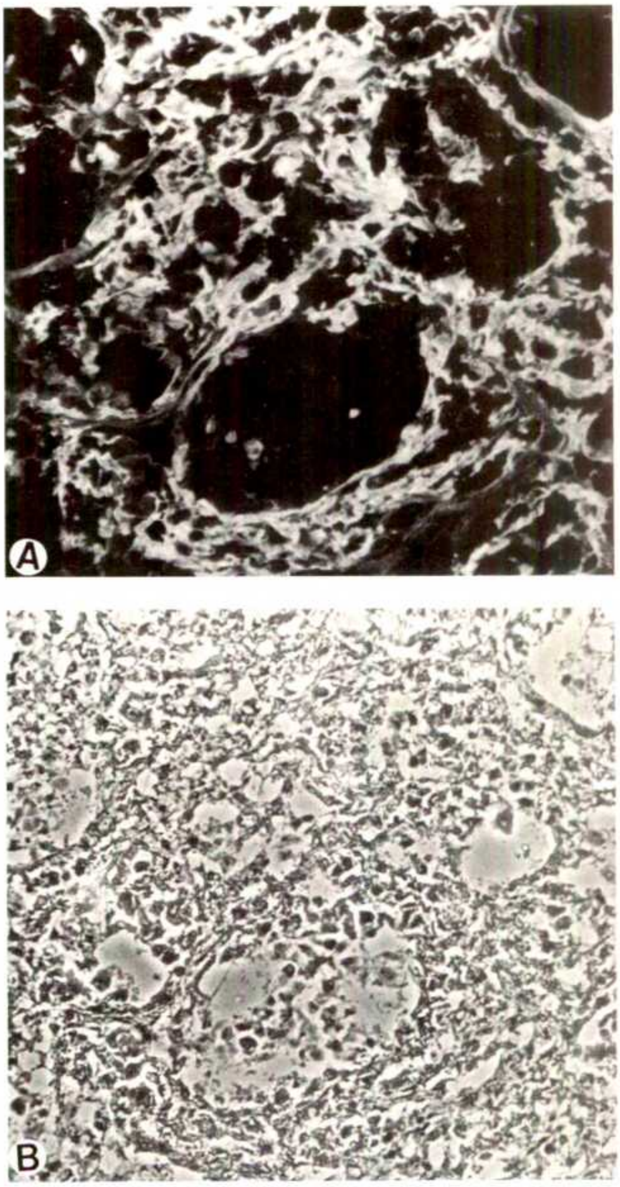

Fig 2. Dense specific fluorescence for fibrin in the connective tissue stroma surrounding extravascular deposits of viable RCC tumor cells observed in a fresh-frozen section by fluorescence (A) and phase-contrast (B) microscopy. Clusters of tumor cells are evident by phase contrast. Fluorescence is lacking within these clusters (original magnification × 160; current magnification × 123).

The endothelial lining of the vascular channels stained with antibody to factor VIII antigen. Little staining of intravascular tumor masses was observed with the antibody to either factor VIII antigen or the platelet IIb-IIIa glycoprotein. Staining that did exist manifested a sparse, nodular pattern. Neither antibody stained structures in extravascular sites. The antibody to platelet IIb-IIIa glycoprotein stained occasional areas in a nodular pattern on the linings of vascular channels as well as peripheral blood platelets.

Antibody to factor VII failed to stain either normal liver or tumor tissue using the fluorescence procedure, but stained both when observed by the ABC technique in sections of fresh-frozen and also formalin-fixed tissue. Factor VII in liver cells was observed in the cytoplasm and also in association with the membranes of hepatic parenchymal cells. Staining in tumor tissue was observed adjacent to the surfaces of tumor cells and also in the intercellular spaces (Fig 3). Using antibody to factor X, we demonstrated factor X in normal liver and in tumor tissue by both fluorescence and peroxidase procedures and in formalin-fixed tissue using the peroxidase procedure. A cytoplasmic pattern was observed in hepatic parenchymal cells, but staining was restricted to the intercellular spaces in tumor tissue (Fig 4). Thus, localization of both factors VII and X was restricted to the pericellular areas around tumor cells. It was not present in perivascular connective tissue or in areas occupied by inflammatory cells.

Staining with antibodies to fibrinogen, factor VIII antigen, platelet IIb-IIIa glycoprotein, factor VII, and factor X was retained in fresh-frozen (unfixed) tissue despite the repeated washings required for these procedures. Staining was not observed when the primary antibody was omitted or when antibodies of other specificities were used. These procedures, together with preincubation with normal blocking serum, which is part of the methodology, preclude nonspecific binding to Fc receptors. Staining with antibodies to factors VII and X, but not with antibody to fibrinogen, was retained in paraffin-imbedded tissue sections. Staining was not observed in normal kidney tissue and was not a consequence of tissue processing. There was no nonspecific tissue staining.

DISCUSSION

RCC is a relatively uncommon tumor[14,15] that is thought to arise from the cells of the proximal convoluted tubule of the nephron.[16] RCC has a striking propensity to thrive in the form of a tumor thrombus within the renal vein and inferior vena cava.[14,15] Venous invasion occurs grossly in 46.2% and histologically in an additional 31% of cases and signals more aggressive behavior of the tumor and a poorer prognosis.[17] RCC tumor cells apparently find conditions within a venous thrombus to their liking since such tumors may achieve considerable proportions, even reaching the right atrium, and may become detached and continue an existence within the vein free of the primary tumor.[18]

Patients with RCC may manifest peripheral blood changes of disseminated intravascular coagulation.[3,19,20] Thromboembolic disease may signal the existence of occult RCC or complicate the course of previously identified disease.[21-24] Sufrin et al[25] found that fibrinogen and fibrin degradation products (FDP) were elevated in RCC, that fibrinogen elevation (but not the FDP or platelet levels) correlated with more advanced disease and a more rapidly progressive course, and that fibrinogen levels declined following nephrectomy. FDP were more commonly increased in patients with metastatic rather than localized disease.[26,27] A functionally abnormal fibrinogen with an increased content of sialic acid has been described in RCC.[28]

A number of coagulation-related studies have been performed on RCC tissue. Clifton and Grossi[29] described a plasminogen activator in explants of RCC tumor tissue. By contrast, Yuen and Kwaan[30] found, in studies of sections of fresh RCC tissue placed on a fibrin plate, that fibrinolysis usually observed about blood vessels in normal tissues was inhibited about vessels in the vicinity of the tumor. This implied that a diffusable inhibitor of fibrinolysis existed within the tumor. O'Meara[31] observed that extracts of normal kidney inserted into blood induced formation of a clot that subsequently lysed. By contrast, introduction of extracts of RCC tissue into blood produced a clot that did not subsequently lyse. Gordon and associates[32] identified a coagulant in RCC tissue that differed from the coagulant of normal tissue in that it did not require factor VII for expression of coagulant activity and it was inhibited by

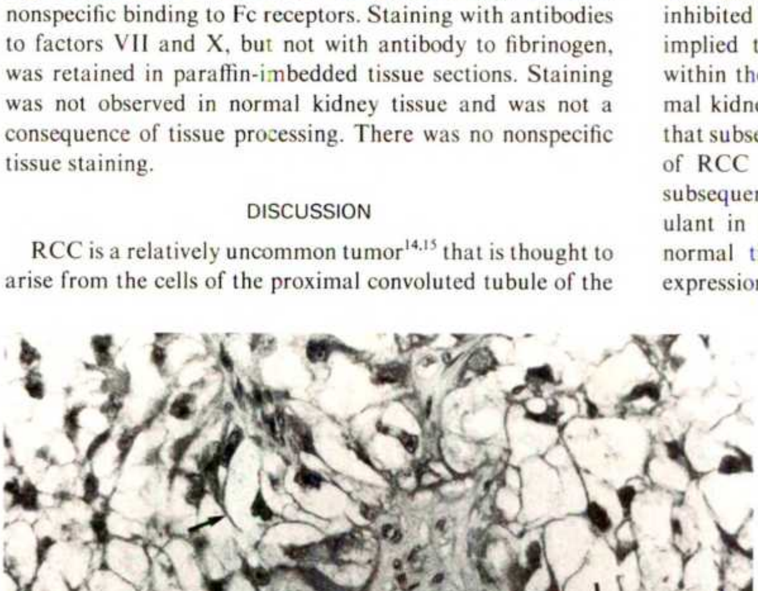

Fig 3. Specific staining by the peroxidase reactions for factor VII in formalin-fixed, paraffin-imbedded RCC tissue. Note the dark precipitate in the pericellular areas outlining individual tumor cells (arrows). In adjoining connective tissue, stained cell nuclei are observed, but cytoplasmic margins are not because the immunospecific staining reaction is not present (hematoxylin counterstain; original magnification × 250; current magnification × 150).

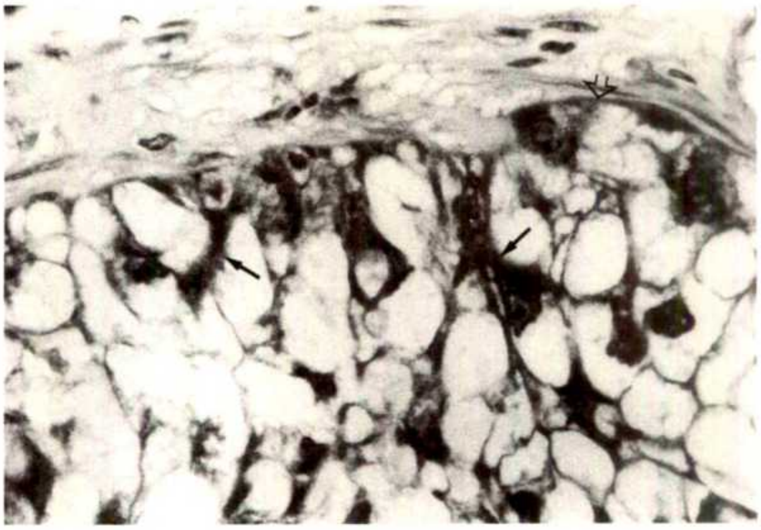

Fig 4. Specific staining by the peroxidase reaction for factor X in formalin-fixed, paraffin-imbedded RCC tissue. Note the dark precipitate in pericellular areas outlining individual tumor cells (arrows). The precipitate is also evident at the tumor-stroma interface (open arrow) but not in adjoining connective tissue (see Fig 3) (original magnification × 400; current magnification × 240).

diisopropylfluorophosphate. For these reasons they claimed that this coagulant was unlike tissue factor (tissue thromboplastin).

Studies by Long et al[33] suggested that RCC tumor cells may manifest coagulative properties in vivo. These authors described a patient with an unresectable RCC involving the renal vein in whom the number of tumor cells within the venous drainage transiently increased during surgery. A subsequent increase in circulating tumor cells was discovered several hours before development of a pulmonary embolism. Two months later the lungs were found by chest x-ray to be studded with metastases.

Naito and colleagues[34] studied the coagulant and fibrinolytic properties of continuous cell lines derived from one patient with rapidly progressive and one patient with slowly progressive RCC. The coagulant corrected the defect in factor IX–deficient plasma and also in factor VII–deficient plasma, but to a lesser extent. The inducer of fibrinolysis produced by these cells was urokinase. Cells from the patient with slowly progressive disease manifested low levels of coagulant and fibrinolytic activity. Upon inoculation of these cells into nude mice, tumors appeared but subsequently regressed. In contrast, cells from the patient with rapidly progressive disease exhibited high levels of coagulant and fibrinolytic activity. Upon inoculation into nude mice, this second cell line demonstrated greater tumorgenicity and more rapid tumor growth than the first cell line.

Steiner[35] found that mouse renal adenocarcinoma cells were potent in vitro inducers of platelet aggregation and release and that platelet lysates promoted mouse renal adenocarcinoma cell growth in culture. Possible platelet aggregating properties of human RCC cell lines have not been described. Sequestration of fibrinogen (tagged with a radiolabeled antibody to fibrinogen) into RCC tumor masses in vivo has been reported in six cases.[36] The effects of coagulation-reactive drugs in RCC have rarely been studied. However, Larsen et al[37] observed no change in the condition of a single patient with RCC treated with a fibrinolytic enzyme, heparin, and aspirin.

In the present study we have documented the occurrence of fibrin associated with extravascular deposits of viable tumor in RCC and also surrounding tumor masses within vascular channels. It is likely that our findings are, in fact, indicative of fibrin. However, the possibility remains that at least a portion of the fluorescence observed with the polyclonal antifibinogen antibody used was due to the presence of fibrinogen that had leaked from blood vessels or of polymerized but non–cross-linked fibrin. Existing literature suggests that tumor cell–associated coagulants may be responsible for initiating the sequence of enzymatic reactions that lead to local fibrin formation through factor VII–independent (cancer procoagulant A–initiated) coagulation pathways.[32] By contrast, the present observations support the possibility that a factor VII–dependent, tissue factor–initiated reaction sequence may be responsible for the fibrin deposited in RCC. Thus, factors VII and X were present extravascularly in the pericellular spaces around tumor cells and adjacent to tumor cell surfaces. These factors were not eliminated from fresh-frozen tissue sections by the repeated washings required for the immunologic techniques used. This suggests that these factors were bound in place and, therefore, possibly in active form. Neither factor VII nor X were present in normal tissue, perivascular connective tissue, or areas occupied by inflammatory cells in tumor tissue. A role for the factor VII–dependent, tissue factor–initiated pathway of fibrin formation would be supported by demonstration of tissue factor antigen on RCC tumor cells. Although such evidence is lacking for RCC, we have previously demonstrated tissue factor on SCCL tumor cells using an antibody to bovine tissue factor that cross-reacted with human tissue factor. Evidence was obtained for platelet deposition on intravascular tumor, but such deposits were smaller than might have been expected from certain experimental studies that have shown platelet masses of substantial proportions associated with tumor cells within blood vessels.[38,39] The extent and significance of platelet involvement in RCC remains undefined.

In summary, evidence presented suggests that activation of the host coagulation mechanism by tumor cells in RCC results in local fibrin formation. Since RCC appears to thrive within a fibrin clot in blood vessels, such clot formation may accommodate tumor growth. Previous studies have shown an abundance of fibrin associated with extravascular tumor deposits in SCCL[9] and that the clinical course of SCCL is ameliorated by warfarin anticoagulation.[4-6] We postulate that tumor progression in RCC may also be interrupted by agents that are capable of limiting local coagulation reactions.

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Correlation of Drug Sensitivity In Vitro With Clinical Responses in Childhood Acute Myeloid Leukemia

By Lois W. Dow, Gary V. Dahl, David K. Kalwinsky, Joseph Mirro, Michael B. Nash, and Paula K. Roberson

Clonogenic cells from 41 children with newly diagnosed acute myeloid leukemia (AML) were tested in vitro for their sensitivity to cytarabine (Ara-C) and daunorubicin (DNR). The findings were then compared with the patients' responses to induction chemotherapy that uniformly included Ara-C and DNR. Light-density marrow cells were incubated with either or both drugs for one hour and cultured over leukocyte feeder layers; clusters and colonies were scored on days 7, 10, and 14. Only the percentage of cell kill in the presence of 1.8 µmol/L DNR was significantly associated with responses to induction therapy: median of 45% (range, 0% to 98%) for patients achieving complete remission *v* 16% (range, 4% to 23%) for nonresponders ($P = .007$). The relationship between clonogenic cell kill ≤23% and clinical responses was striking. Of the 11 evaluable patients with in vitro findings in this category, ten either failed induction therapy or relapsed within 1 year after attaining remission. Kaplan-Meier analysis of relapse-free survival times indicated longer durations of remission for patients whose blast cells showed increased sensitivity in vitro to Ara-C alone, DNR alone, or a combination of the two agents. Seven of 11 patients with cell kills of ≥49% in the presence of 1.25 µmol/L Ara-C remain free of leukemia, compared with only one of 12 whose cells were less sensitive to the drug ($P = .006$). We conclude that the in vitro sensitivity of clonogenic leukemic progenitors to DNR and Ara-C correlates with treatment outcome in children with newly diagnosed AML.
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CLONOGENIC ASSAYS of malignant stem cells have been proposed as a method for obtaining reliable information about the chemosensitivity of a patient's tumor cells and evaluating the probable clinical efficacy of anticancer therapy.[1-4] In the human leukemias, normal progenitor cells are replaced by abnormal progenitors with a spectrum of proliferative potentials.[5,6] Such cells from patients with acute myeloid leukemia (AML) usually form clusters or colonies in vitro when stimulated by leukocyte feeder layers or conditioned media. Preisler and others[3,7-11] have reported that the in vitro drug sensitivity of clonogenic progenitors from different groups of adult patients with AML studied at diagnosis and relapse correlates with clinical responses to various induction regimens. Interpretation of these findings has been complicated by the lack of uniform treatment of blast cells in vitro and in vivo and by inclusion of patients who were studied after relapse as well as at diagnosis. To estimate the predictive value of clonogenic assays in childhood AML, we compared the in vitro sensitivity of cells to cytarabine and daunorubicin with induction responses to the same drugs. Additional comparisons were done to find whether sensitivity to these agents would reliably predict the long-term outcome of therapy.

PATIENTS AND METHODS

The study comprised 41 patients with newly diagnosed AML from whom sufficient cells for cultures were obtained during the period from April 1980 through Oct 1983. The distribution of ages, races, initial leukocyte and platelet counts, blast cell morphologic types and cytogenetic features among the study group did not differ significantly from that among 43 other patients who were treated according to the same therapeutic protocol. Responses to induction therapy and relapse-free survival times in the two groups were also similar (median times to failure, 293 and 341 days, excluding patients who received bone marrow transplants).

There were 27 boys and 14 girls, with ages ranging from 4 months to 19.3 years (median, 6 years). Thirty-six patients were white, and five were nonwhite. Leukocyte counts at diagnosis ranged from 1.6 to 400 × 10^9/L (median, 27.6 × 10^9/L). By the French-American-British (FAB) classification system,[12] 8 patients had M_1 disease, 14 M_2, 3 M_3, 12 M_4, and 4 M_5. Cells from 17 children had Auer rods. Analysis of the patients' marrow cell karyotypes revealed normal ploidy in 16 cases, a combination of normal and abnormal ploidy in nine, and abnormal ploidy only in 13. Three children had inadequate chromosome preparations. Chromosomal translocations, including the t(8;21), were identified in 13 patients. One child had monosomy 7, and two had an inversion of chromosome 16 (p13;q22). These investigations were performed with the informed consent of the patients or their parents and with the approval of the Clinical Trials Committee of St Jude Children's Research Hospital.

Outline of therapy. The treatment protocol[13] is summarized in Fig 1. Each child initially received three daily doses of daunorubicin (DNR) followed by a seven-day continuous infusion of cytarabine (Ara-C). If blasts were present in a marrow aspirate obtained on day 14, a second course of DNR/ara-C was given immediately. Patients with marrow hypoplasia and <5% blasts received a second course of DNR/Ara-C after marrow recovery. For this analysis, patients who entered hematologic remission after the first or second course of therapy were considered responsive to induction therapy. Those with residual leukemia or bone marrow recovery with leukemic blasts after two courses of induction therapy were classified as nonresponders. Patients who died during induction therapy without marrow recovery or failed to complete the protocol-specified therapy were excluded from this analysis.[3] Six patients who achieved a complete remission and had a suitable marrow donor were given a bone marrow transplant. Two patients achieved hematologic but not complete remission; all others entered complete remission and received monthly cycles of chemotherapy as outlined in Fig 1 and were evaluated for duration of complete remission.

Cell culture methods and drug sensitivity testing. Aspirated bone marrow cells obtained at diagnosis were collected in preservative-free heparin. Light-density cells (<1.077 g/mL) were separated by density centrifugation with Ficoll-metrizoate,[14] yielding 58% to 100% blasts (median 96%). These were incubated for 60 minutes

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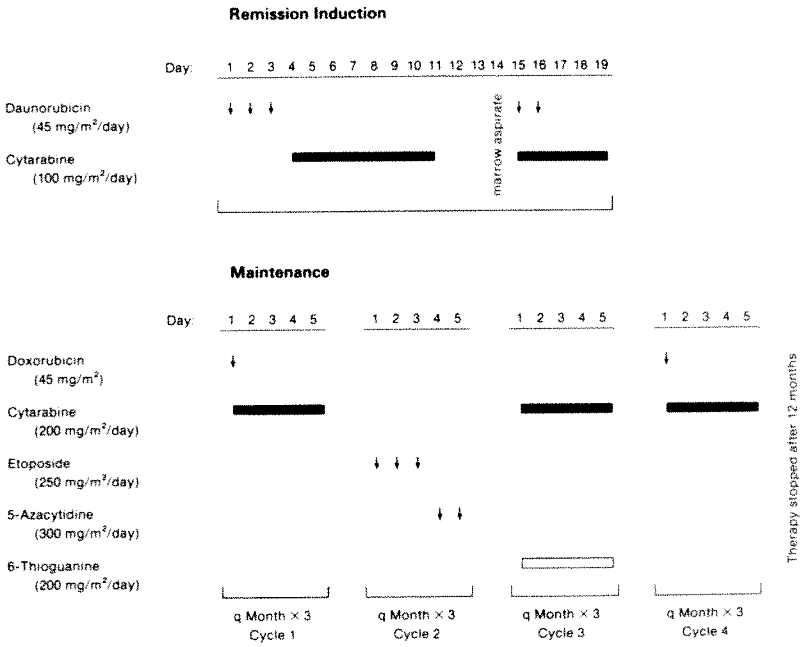
Remission Induction
Day: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Daunorubicin (45 mg/m²/day)
Cytarabine (100 mg/m²/day)
marrow aspirate
Maintenance
Day: 1 2 3 4 5
Doxorubicin (45 mg/m²)
Cytarabine (200 mg/m²/day)
Etoposide (250 mg/m²/day)
5-Azacytidine (300 mg/m²/day)
6-Thioguanine (200 mg/m²/day)
q Month × 3 Cycle 1
q Month × 3 Cycle 2
q Month × 3 Cycle 3
q Month × 3 Cycle 4
Therapy stopped after 12 months

Fig 1. Schema of therapy. All patients received at least one course of DNR/Ara-C. If the marrow aspirate obtained on day 14 contained greater than 5% blasts, a second course of the same agents was given immediately. For children with hypoplastic marrows (≤5% blasts), the second course was delayed until marrow recovery occurred. Patients who attained remission and had an HLA-mixed lymphocyte culture (MLC)–compatible marrow donor received a bone marrow transplant. Those without a suitable donor received monthly cycles of continuation therapy, as shown in the figure. All postinduction treatment was stopped after 1 year.

(1×10^6 cells/mL) with 1.8 μmol/L DNR, 1.25 μmol/L Ara-C, and 12.5 μmol/L Ara-C or 1.25 μmol/L Ara-C plus 1.8 μmol/L DNR as reported by Preisler.[3] When sufficient cells were available, additional incubations with drug for 18 hours were performed. The cells were then washed and plated in quadruplicate in 35-mm Petri dishes (Miles Scientific, Naperville, Ill) at 2×10^5cells/mL in 0.3% agar with a leukocyte feeder layer.[5,15] Cultures were incubated in a 7.5% CO_2 atmosphere at 37 °C, and clusters of more than three cells and colonies of >50 cells were scored on days 7, 10, and 14. The percentage of cell kill was determined by dividing the number of clusters and colonies of drug-exposed clonogenic cells (L-CFU) by that for cultures of untreated L-CFU and subtracting the result from 100%. Only studies for which control plates had 20 or more clusters and colonies were included in the analysis. In four of 144 drug assays, greater growth was noted for drug-exposed cells than for controls, resulting in negative cell kill; these values appear as zero in Fig 2.

Cells obtained from 46 of 72 patients, or 64%, met the criteria for drug sensitivity testing. However, five of the 46 presented prior to the beginning of in vitro drug sensitivity studies, leaving 41 eligible for testing. The numbers of clusters and colonies formed by clonogenic cells from 41 patients in this study ranged from 23 to 3,088, with a median of 538 per 2×10^5 cells plated. Cells from 17 of these patients formed more than 19 large clusters and colonies, and cells from 24 formed predominately small clusters.

The 43 patients not studied for drug sensitivity in vitro included the five patients cited in the preceding paragraph, three with insufficient cell numbers, 12 from whom samples were not collected, and 23 having fewer than 20 clusters or colonies on day 7. A comparison of the latter 23 patients to the 41 whose cells produced clusters and colonies in vitro revealed no significant difference in either the proportion achieving remission or the duration of remission. Growth patterns (cluster/colony ratios) were not related to response.

When clonogenic progenitors were scored on days 10 and 14, cell growth for the majority of patients had decreased (L.W. Dow, unpublished observation). The data are therefore reported for day 7.

Statistical methods. Mann-Whitney tests and chi-square tests were used to compare clinical characteristics between patients with and without cell culture results. The percentages of cells killed by Ara-C and DNR for responding and nonresponding patients were compared with use of Mann-Whitney tests. In the statistical analysis, patients were considered to have resistant disease if they failed to achieve remission after two cycles of DNR and Ara-C; additional analysis extended the definition of clinical drug resistance to cases in which relapse occurred within one year from the date of remission. Relapse-free survival curves were constructed by the Kaplan-Meier procedure, and significant differences between results were determined by the log rank test.[16,17] Two patients in hematologic but not complete remission and six who received a bone marrow transplant were excluded from the Kaplan-Meier analysis. A method based on the log rank test was used to identify percentages of cell kill that yielded maximal differences in remission durations. The potential influence of various clinical and laboratory variables on duration of continuous complete remission was evaluated in a stepwise multivariate analysis using the model of Cox.[18]

RESULTS

Thirty-one of the 41 patients whose blast cells were studied for chemosensitivity in vitro achieved a complete or hematologic remission after one or two courses of DNR and Ara-C, six had resistant disease requiring alternative therapy, and four could not be evaluated for clinical responses because of early death. Of these four, one died early in induction therapy (day 4); two others died on days 18 and 36 of intracranial hemorrhages without evidence of leukemia; and the fourth, who had monosomy 7, died with prolonged marrow hypoplasia and disseminated aspergillosis after two cycles of induction therapy.

Figure 2 shows the percentages of cell kill for clonogenic progenitors after one hour of incubation with either DNR or Ara-C, or both drugs, as determined from cluster and colony formation on day 7. The results obtained with 1.8 μmol/L DNR alone (panel A) were significantly related to clinical responses: 45% median cell kill (range, 0% to 98%) for patients entering remission *v* 16% (range, 4% to 23%) for

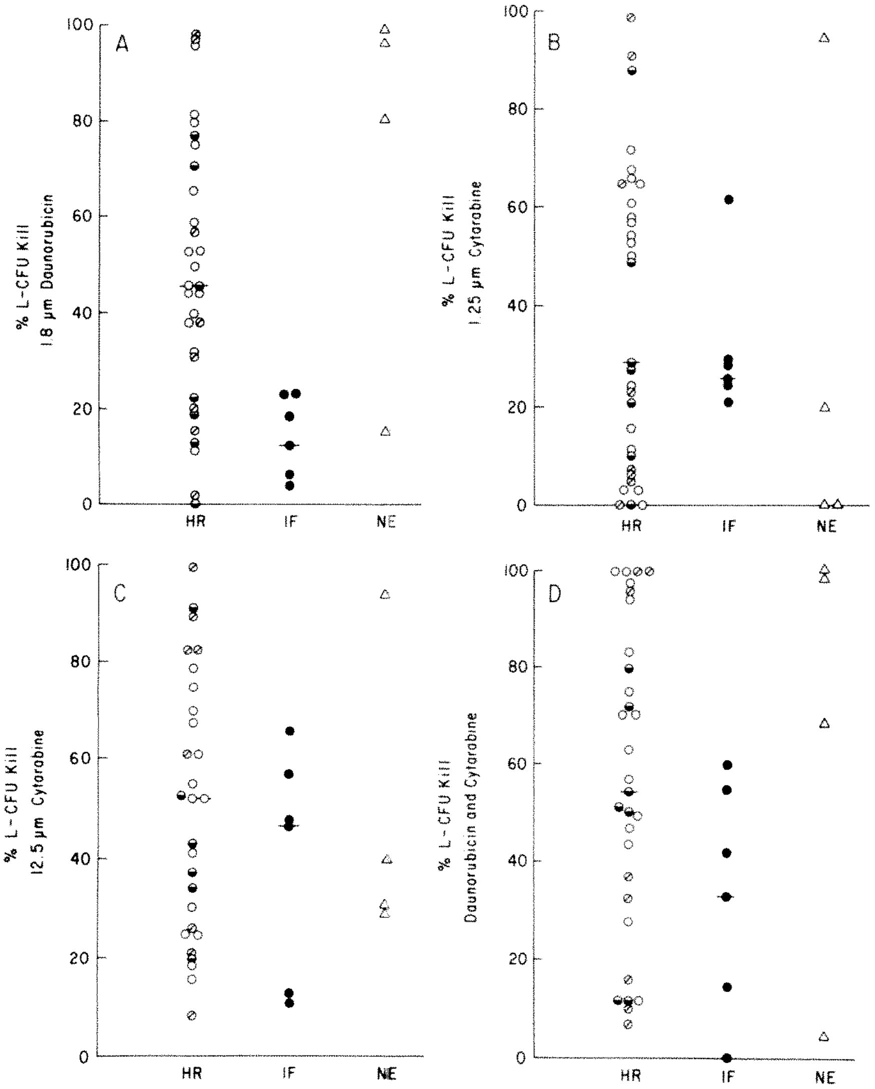

Fig 2. (A-D) Relationship of clonogenic cell kill in vitro to clinical responses. The cells were incubated with the indicated drug for one hour, and cluster and colony formation was scored on day 7. Abbreviations: HR, hematologic remission; IF (●), induction failure due to resistant disease, NE (△), nonevaluable for induction response. In the HR column, ○ designates patients in remission for ≥1 year, ⊘ designates in remission but not evaluable for remission duration, and ◒ designates patients in remission for <1 year. Bars indicate median values.

induction failures resulting from persistent leukemia ($P = .007$). Closer examination of our data revealed that low levels of chemosensitivity, as measured by the clonogenic assay, predicted clinical drug resistance with greater than 90% accuracy. Of 11 patients with in vitro cell kills of ≤23%, ten either failed induction therapy or relapsed within 1 year after attaining remission. At higher levels of cell kill, only three of 18 patients failed to achieve at least 1 year of remission.

The chemosensitivity of cells exposed to 1.25 or 12.5 µmol/L Ara-C alone or to Ara-C and DNR in combination did not achieve statistical significance as a predictor of induction responses. However, 11 of 19 patients with cell kills ≤49% with 1.25 µmol/L Ara-C failed therapy within 1 year, compared with two of 11 with higher percentages. With a tenfold higher concentration of Ara-C, nine of 17 patients with cell kills of ≤54% failed early, compared with three of nine whose blasts showed greater chemosensitivity. Combining Ara-C (1.25 µmol/L) with DNR (1.8 µml/L) did not improve the results obtained with either Ara-C or DNR alone.

Twenty-three of the 31 patients who achieved remission were eligible for assessment of in vitro chemosensitivity as a predictor of remission duration. Kaplan-Meier analysis was performed for groups of patients defined by percentages of cell kill yielding maximal differences in remission durations (Fig 3). With a median follow-up time of 35 months, only three of nine patients with an in vitro cell kill of ≥53% in the presence of DNR have relapsed, compared with 12 of 14 with lower percentages ($P = 0.010$). Similarly, only four of 11 patients with ≥49% kill of progenitors exposed to 1.25 µmol/L Ara-C have failed therapy, compared with 11 of 12 with lower percentages ($P = .006$). Incubation of cells with 12.5 µmol/L Ara-C or a combination of DNR and Ara-C also yielded groups with significantly different relapse rates (P values = .037 and .034, respectively). Importantly, median lengths of remission for patients with higher cell kill in the presence of 1.8 µmol/L DNR, 1.25 µmol/L Ara-C, 12.5 µmol/L Ara-C (data not shown), or a combination of DNR and Ara-C exceed 693 days and cannot be estimated, whereas those for the corresponding groups defined by lower levels of cell kill do not exceed 495 days.

To further assess the relationship of in vitro kill and other possible prognostic factors to remission duration, we per-

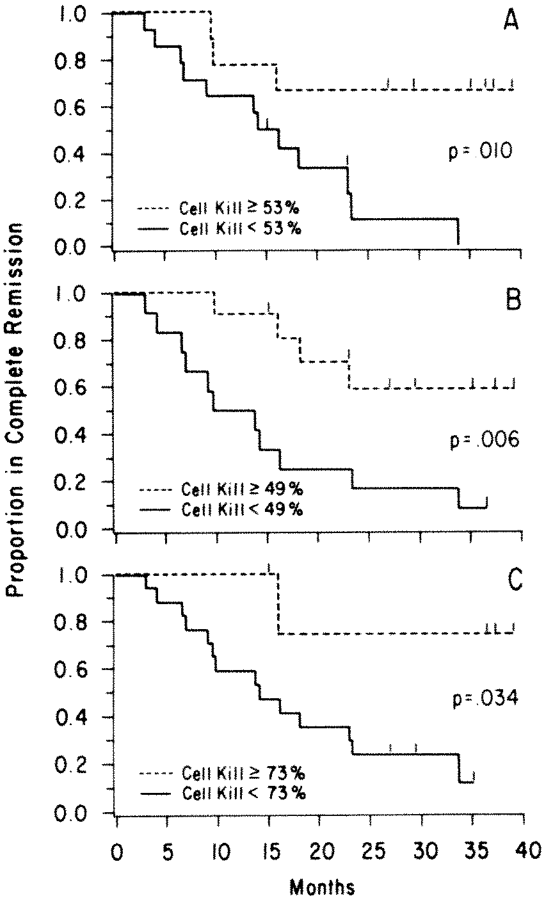

Fig 3. Kaplan-Meier plots of complete remission durations according to level of in vitro cell kill. The values that defined the different patient groups were those yielding maximal differences in relapse-free survival. Marrow cells were incubated with (A) 1.8 μmol/L DNR, (B) 1.25 μmol/L Ara-C, or (C) 1.25 μmol/L Ara-C and 1.8 μmol/L DNR. Differences between the curves were compared statistically with the log rank test.

formed a Cox regression analysis. Variables included age, leukocyte count ($<25 \times 10^9$/L v $\geq 25 \times 10^9$/L), presence or absence of CNS leukemia at diagnosis, FAB morphologic classification (M_1 to M_3 v M_4 to M_5), and percentage of cell kill in vitro with each drug or the combination of Ara-C and DNR. Only the cell kill by DNR in vitro was related to remission duration ($P = .04$).

To test the specificity of cell kill in vitro by DNR and Ara-C, we divided the patients into groups based on the same percentages listed in Fig 3. Twenty of 25 patients with low cell kill by DNR in vitro had a low cell kill with 1.25 μmol/L Ara-C, whereas ten of 12 with a high DNR-induced cell kill also had a high cell kill with Ara-C. Similar relationships were seen when DNR was compared with the tenfold higher concentration of Ara-C and when either drug as a single agent was compared with a combination of the two drugs.

We also examined the relationship of ploidy to in vitro drug sensitivity to each of these agents, outcome of therapy, and to growth patterns in vitro. There were no demonstrable correlations (analysis not shown).

Twenty-three patients had sufficient cells for in vitro assessment of cell kill after incubation with Ara-C for 18 hours prior to plating, and 22 could be evaluated for response to DNR after a similar time of drug exposure. Cell kill in the 18-hour assays did not correlate with response to induction therapy; however, only four patients with resistant disease had sufficient cells for study (Fig 4). Among our smaller number of evaluable patients, remission durations were related to in vitro cell kill after an 18-hour incubation with DNR but not Ara-C. Only two of seven patients with cell kills greater than 41% in the presence of DNR have relapsed, compared with six of six patients having lower percentages ($P = .03$).

DISCUSSION

Because clinical features have generally failed to predict responses to therapy in childhood AML,[19] we sought in vitro measures of the chemosensitivity of the patients' leukemia cells. The stem cell model of human cancer predicts that clinical responses to treatment should relate only to the killing of cells with clonogenic potential. Recent observations of Preisler,[3] Park et al,[7] Browman et al.[8] and others[9-11] indicate that in vitro assays of cluster and colony formation by malignant progenitor cells will predict clinical drug resistance in a high proportion of cases; however, for children with newly diagnosed AML, it has not been demonstrated that the chemosensitivity of clonogenic progenitors correlates with treatment outcome.

Our study is the first in which clonogenic progenitor cells obtained only at diagnosis were exposed to the same agents used for remission induction. Results of the assay indicate that the in vitro sensitivity of leukemic progenitors to DNR, but not Ara-C, is highly correlated with subsequent responses to induction therapy. This suggests a greater therapeutic contribution from the anthracycline, consistent with the higher remission induction rates reported for DNR as a single agent in AML in doses similar to this regimen.[20] That the duration of complete remission correlates with percent DNR cell kill in vitro also suggests the relationship between in vitro and in vivo cell kill.

Typically, an assay that predicts clinical drug resistance is correct in 90% of cases, whereas the prediction of drug sensitivity is correct in 40% to 70% of cases.[21] When induction responses were used as the end point, drug resistance in the assay (ie, cell kill of $\leq$23%) was associated with clinical resistance in 55% of the patients. However, if both failure to achieve remission and early development of recurrent disease, within 1 year from the date of remission, were considered evidence of drug resistance, cell kill by DNR in vitro met the 90% criterion for a clinically useful predictor of drug efficacy.

The lack of a statistically significant relationship between cell kill by Ara-C, with or without addition of DNR, and clinical responsiveness to induction therapy conflicts with previous studies of clonogenic assays in AML. Preisler,[3] for example, has reported that the sensitivity or resistance to either Ara-C or DNR in vitro correlated with the induction responses of 25 older patients with newly diagnosed or relapsed AML, some with leukemia following cytotoxic treatment for other malignancies. Similar findings were

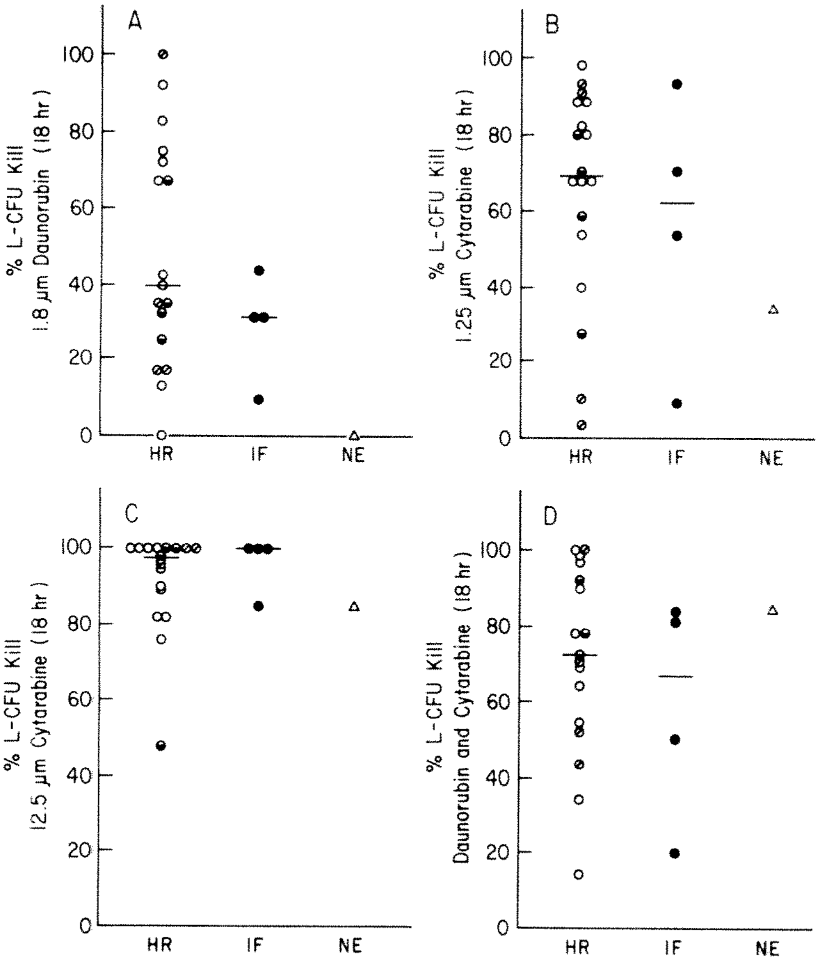

Fig 4. Relationship of clinical responses and clonogenic cell kill after 18-hour incubations with drugs (A-D). The data are reported as in Fig 2.

reported by Browman et al[8] for 38 adults treated with Ara-C and an anthracycline. Using different methods, Gustavsson and Olofsson,[11] Lihou and Smith,[9] and Park et al[7] also found a positive correlation between the sensitivity of blast cells to Ara-C and anthracyclines in vitro and induction responses in mixed groups of newly diagnosed and relapsed patients. Finally, Marie et al[10] demonstrated a correlation with the outcome of induction therapy for Ara-C but not doxorubicin in studies in which clonogenic cells were exposed continuously to drugs. These discrepancies may reflect different assay methods, different criteria for the inclusion of patients in the studies, or perhaps inherently different leukemic stem cells in childhood *v* adult AML.[22]

That in vitro kill with DNR alone differs from the kill with simultaneous exposure to Ara-C and DNR may be related to the cytotoxic effects of each drug on a patient's cells in vitro. Fried et al[23,24] have shown that the cytotoxic effects of a one-hour simultaneous exposure of Chinese hamster ovary cells to Ara-C and DNR are related to the relative effects of each drug in the combination. When a moderately cytotoxic dose of DNR and a less cytotoxic dose of Ara-C were used, the observed cell kill with the combination was less than expected. When a higher, more cytotoxic concentration of Ara-C was coupled with a less cytotoxic dose of DNR, the results equaled the expected cytotoxicity. In their experiments, relatively low Ara-C concentrations inhibited cell cycle progression and provided partial protection from DNR cytotoxicity.

Methods of studying the sensitivity of leukemic cells to phase-specific antimetabolites such as Ara-C are controversial. Such studies have been complicated by the rapid deamination of the drug and its Ara-CMP (Ara-C monophosphate) metabolite. Preisler[3] has recommended relating the sensitivity of clonogenic progenitors in S-phase to Ara-C by use of a thymidine suicide index to distinguish kinetically resistant from biochemically resistant cells. However, our data (not shown) do not indicate a relationship between the sensitivity of S-phase cells to Ara-C and the results of induction therapy or duration of remission. Lihou and Smith[9] have recommended continuous exposure of cultures to Ara-C to obtain results more representative of the clinical situation, whereas Park et al[7] has refed cultures daily, adding Ara-C as well as nutrients. We exposed clonogenic progenitors from 22 patients to Ara-C for 18 hours before plating and, despite greater cell kill, found no correlation with induction responses.

Weisenthal and Lippmann[25] have suggested that the results of clonogenic assays reflect the general chemosensitivity of the proliferating leukemic cells in vivo rather than their responsiveness to particular agents. This hypothesis has been difficult to test in AML since most studies with clonogenic assays have not been performed in conjunction with a prospective clinical trial. That the sensitivity of clonogenic progenitors to DNR or Ara-C alone, or to a combination of both drugs, showed a relationship to remission durations among our patients would support the idea of Weisenthal and Lippman. However, the failure of cell survival in the presence of Ara-C, even with added DNR, to predict early development of drug resistance suggests that the early responses of patients to chemotherapy may be related to the activity of specific drugs such as DNR. This is supported by the correlation of in vitro kill by DNR and remission duration. Alternatively, the separation of patients into favorable and unfavorable groups based on maximal differences in relapse-free survival could have increased the probability of false-positive results so that Ara-C might appear to have prognostic value. Further research comparing dose-response curves for AML cells exposed to different classes of antileukemic agents is needed to resolve this question.

The ability of the clonogenic assay to select patients who are most likely to fail anthracycline-based induction therapy or to relapse early should be confirmed in a prospective trial and exploited in treatment planning. Alternative methods may extend the prediction of clinical drug sensitivity to agents such as Ara-C and lead to clinically relevant models of drug combinations.

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Acute Lymphoblastic Leukemia With Unusual Cytoplasmic Granulation: A Morphologic, Cytochemical, and Ultrastructural Study

By Jean Fradera, Enrique Vélez-García, and James G. White

The classification of the acute leukemias depends mainly on the morphologic and cytochemical evaluation of the blast forms. One of the main accepted morphologic criteria in the differentiation between acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML) is the absence of granules in the blast cells of ALL. We evaluated a patient with ALL in whom granules were present in the cytoplasm of 35% of the blast cells, as seen in AML. Cytochemical evaluation was performed, including periodic acid–Schiff reaction, Sudan black B, α-naphthyl acetate, α-naphthyl butyrate, naphthol AS-D chloroacetate, and acid phosphatase stains. The results of these studies confirmed the morphologic impression and diagnosis of ALL. Ultrastructural evaluation revealed that the granules consisted of many tiny vesicles closely packed together in a proteinaceous matrix, resembling to some extent the inclusions described in lymphocytes in the Chédiak-Higashi syndrome, but clearly different. The morphologic, cytochemical, and ultrastructural studies of this unique case are presented in detail. To our knowledge, this is the first time that such granules have been described in blast cells of ALL.

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We have studied a patient with ALL in whom granules were present in the cytoplasm of the blasts, as seen in AML. The morphologic, cytochemical, and electron microscopic studies performed on these blasts have been remarkable and represent to our knowledge the first report of such cytoplasmic granulation in lymphoblasts.

CASE REPORT

A 45-year-old white female with a negative past medical history presented with right upper-quadrant pain. Physical examination revealed hepatomegaly with the border palpated 7 cm below the right costal margin. There was no splenomegaly or lymphadenopathy. The hemogram was as follows: hemoglobin level, 14.7 g/dL; WBC count, 158,000/μL; differential cell count, 78% blasts, 3% segmented neutrophils, 4% bands, and 15% mature lymphocytes; and platelet count, 264,000/μL. Many of the blast cells had large cytoplasmic granules, the rest were typical lymphoblasts without granulation. The blood chemistries (Sequential Multiple Analyzer–12, Techicon) were normal. A spinal tap yielded cerebrospinal fluid that was free of cells. The chest roentgenogram was unremarkable; liver and spleen scans revealed mild hepatosplenomegaly, and the serum electrophoresis showed slight hyperalbuminemia without other abnormalities.

The bone marrow was markedly infiltrated with typical lymphoblasts, and mild mature lymphocytosis was also present. The diagnosis of ALL, L_2 using the French-American-British classification[1] was made in spite of the presence of the blast forms with coarse granulation. Cytochemical studies were performed that confirmed this diagnosis.

Treatment was instituted with vincristine and prednisone after informed consent was given by the patient, and complete remission was obtained 6 weeks later. Bone marrow aspiration at this time revealed marked mature lymphocytosis of uncertain significance. Prednisone therapy was tapered, but shortly thereafter, the patient relapsed and was given vincristine and prednisone again with an immediate response. Treatment was continued for 6 weeks, after which prednisone was tapered again. Complete remission was confirmed by bone marrow aspiration, and maintenance chemotherapy with methotrexate and 6-mercaptopurine was begun with the consent of the patient and continued until the patient relapsed again 3 months later. Reinduction with vincristine and prednisone was unsuccessful this time, and treatment with doxorubicin and arabinosyl cytosine was instituted.

CNS involvement was confirmed and treated with cranial radiation. Sepsis ensued, and the patient was treated aggressively with antibiotics including cephalosporins and aminoglycosides. Antifungal agents were administered for systemic candidiasis, and platelet and granulocyte transfusions were given with poor response. Multiple complications occurred, including hepatitis, which precluded further therapy. Supportive treatment was continued until death supervened from complications approximately 1 year after the initial diagnosis of acute leukemia.

MATERIALS AND METHODS

Blood smears and cytochemical studies. The peripheral blood smears and bone marrow preparations were stained with Wright's-Giemsa stain. The cytochemical studies performed included Sudan black B stain, periodic acid–Schiff reaction (PAS), naphthol AS-D chloroacetate esterase, α-naphthyl acetate esterase, α-naphthyl butyrate esterase, and acid phosphatase.

Electron microscopic studies. Blood for this study was obtained from our patient with lymphoblastic leukemia and from one child with the Chédiak-Higashi syndrome. Samples were obtained by venipuncture and mixed immediately with citrate–citric acid–dextrose, pH 6.5 (9.3 mmol/L sodium citrate, 7.0 mmol/L citric acid, and 140 mmol/L dextrose) in the ratio of nine parts blood to one part anticoagulant. The blood was centrifuged at 100 g for 20 minutes at room temperature. Platelet-rich plasma was aspirated above the buffy coat. Leukocyte-rich buffy coat was then separated from the red cell layer by careful aspiration and transferred to a second tube. The buffy coats from several tubes were combined and mixed with an equal volume of 0.1% glutaraldehyde in White's saline, pH 7.3 (a 10% solution of a 1:1 mixture of 2.4 mol/L NaCl, 0.1 mol/L KCl, 46 mmol/L $MgSO_4$, 64 mmol/L $Ca(NO_3)_2 \cdot 4H_20$, 0.13 mol/L $NaHCO_3$, 8.4 mmol/L $NaH_2PO_4 \cdot 7H_2O$, 3.8 mmol/L

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The bone marrow was markedly infiltrated with typical lymphoblasts, and mild mature lymphocytosis was also present. The diagnosis of ALL, L_2 using the French-American-British classification[1] was made in spite of the presence of the blast forms with coarse granulation. Cytochemical studies were performed that confirmed this diagnosis.

Treatment was instituted with vincristine and prednisone after informed consent was given by the patient, and complete remission was obtained 6 weeks later. Bone marrow aspiration at this time revealed marked mature lymphocytosis of uncertain significance. Prednisone therapy was tapered, but shortly thereafter, the patient relapsed and was given vincristine and prednisone again with an immediate response. Treatment was continued for 6 weeks, after which prednisone was tapered again. Complete remission was confirmed by bone marrow aspiration, and maintenance chemotherapy with methotrexate and 6-mercaptopurine was begun with the consent of the patient and continued until the patient relapsed again 3 months later. Reinduction with vincristine and prednisone was unsuccessful this time, and treatment with doxorubicin and arabinosyl cytosine was instituted.

CNS involvement was confirmed and treated with cranial radiation. Sepsis ensued, and the patient was treated aggressively with antibiotics including cephalosporins and aminoglycosides. Antifungal agents were administered for systemic candidiasis, and platelet and granulocyte transfusions were given with poor response. Multiple complications occurred, including hepatitis, which precluded further therapy. Supportive treatment was continued until death supervened from complications approximately 1 year after the initial diagnosis of acute leukemia.

MATERIALS AND METHODS

Blood smears and cytochemical studies. The peripheral blood smears and bone marrow preparations were stained with Wright's-Giemsa stain. The cytochemical studies performed included Sudan black B stain, periodic acid-Schiff reaction (PAS), naphthol AS-D chloroacetate esterase, α-naphthyl acetate esterase, α-naphthyl butyrate esterase, and acid phosphatase.

Electron microscopic studies. Blood for this study was obtained from our patient with lymphoblastic leukemia and from one child with the Chédiak-Higashi syndrome. Samples were obtained by venipuncture and mixed immediately with citrate-citric acid-dextrose, pH 6.5 (9.3 mmol/L sodium citrate, 7.0 mmol/L citric acid, and 140 mmol/L dextrose) in the ratio of nine parts blood to one part anticoagulant. The blood was centrifuged at 100 g for 20 minutes at room temperature. Platelet-rich plasma was aspirated above the buffy coat. Leukocyte-rich buffy coat was then separated from the red cell layer by careful aspiration and transferred to a second tube. The buffy coats from several tubes were combined and mixed with an equal volume of 0.1% glutaraldehyde in White's saline, pH 7.3 (a 10% solution of a 1:1 mixture of 2.4 mol/L NaCl, 0.1 mol/L KCl, 46 mmol/L $MgSO_4$, 64 mmol/L $Ca(NO_3)_2 \cdot 4H_2O$, 0.13 mol/L $NaHCO_3$, 8.4 mmol/L $NaH_2PO_4 \cdot 7H_2O$, 3.8 mmol/L

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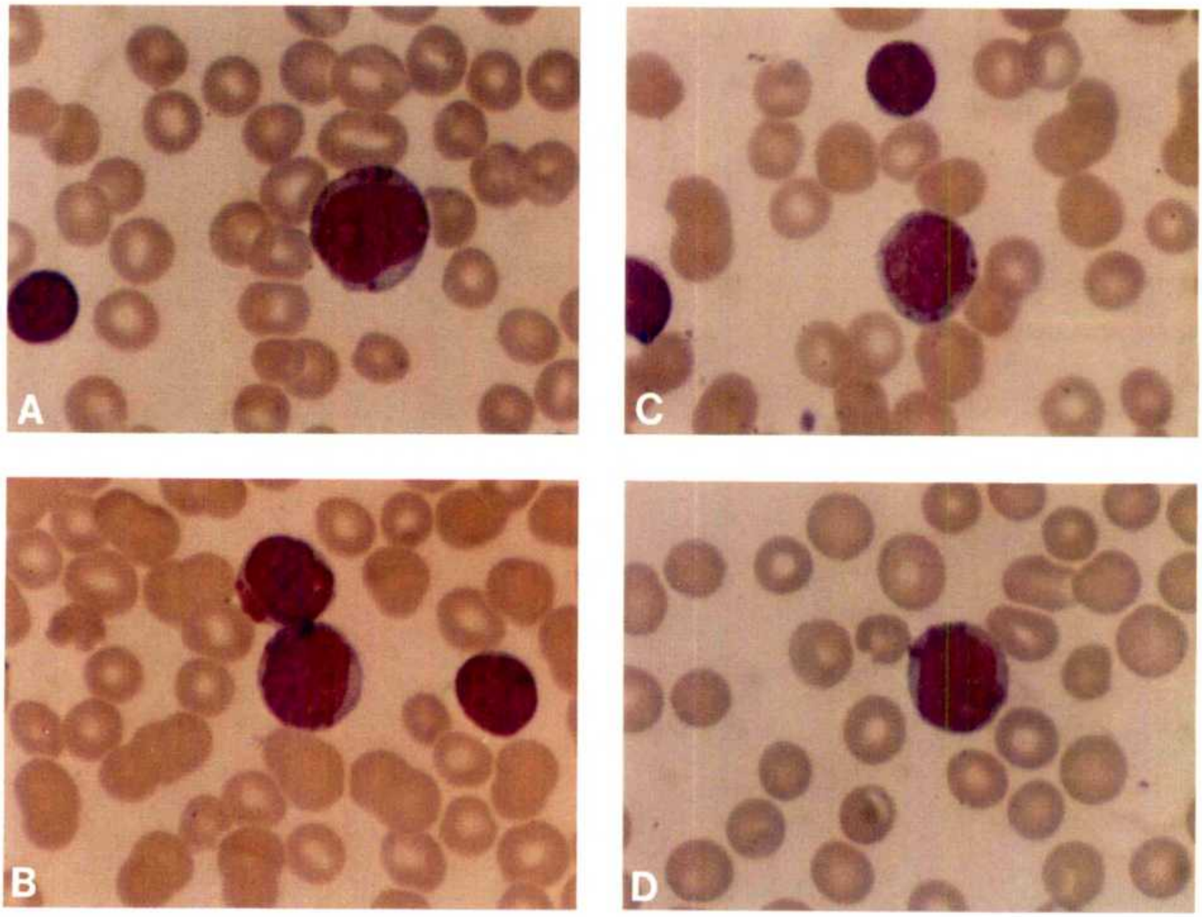

Fig 1. Peripheral blood from the patient illustrating lymphoblasts with the abnormal large cytoplasmic granules. Some of the lymphoblasts have slightly indented nuclei with prominent nucleoli (A, B). Although in all the lymphoblasts with granulation separate distinct granules were identified (C, D), in some of them fusion of several granules could also be observed (A, B, D). Wright's-Giemsa stain (original magnification × 1000; current magnification × 920).

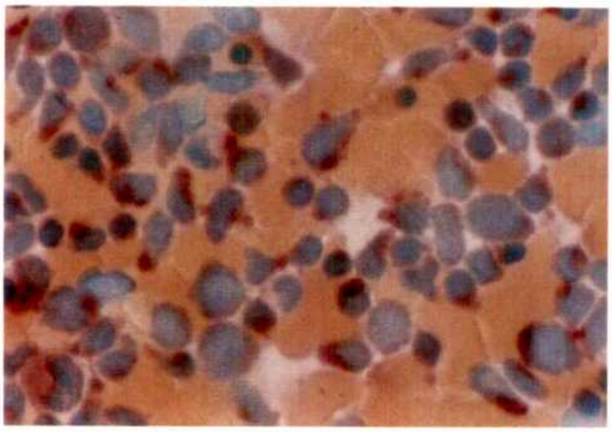

Fig 2. Patient's bone marrow smear illustrating the positive reaction obtained with the α-naphthyl acetate stain in the lymphoblasts (original magnification × 1000; current magnification × 630).

tive disorders of T cell lineage. At the present time a distinct subtype of chronic lymphocytic leukemia (CLL) is recognized in which mature lymphocytes with abundant cytoplasm and large azurophilic granules are frequently observed. Characteristically, these lymphocytes have a strong, tartrate-inhibited positive reaction, with the acid phosphatase stain mainly localized paranuclearly.[10] On electron microscopy, the granules closely resemble mitochondria morphologically; therefore, their origin from the latter cannot be excluded.

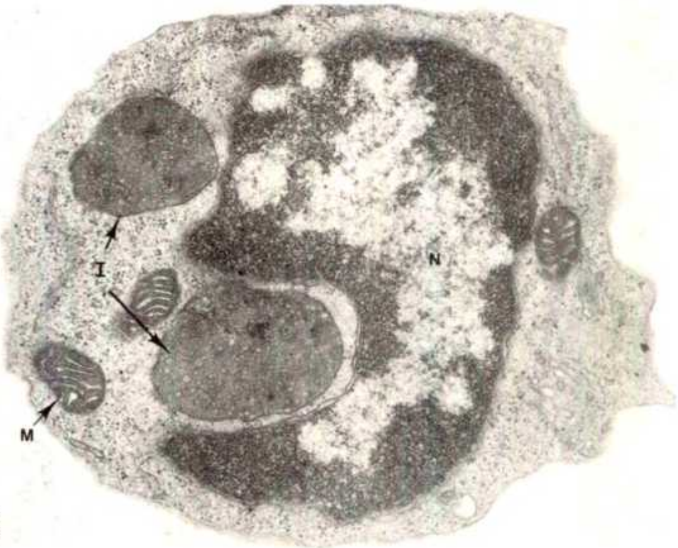

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Fig 3. Thin section of lymphoblast from example of peripheral blood obtained from patient described in this study and prepared for electron microscopy by fixation in glutaralehyde and osmic acid. Nucleus (N), mitochondria (M), and cytoplasm demonstrate typical lymphoid features. Two large inclusions (I) manifesting a vesicular substructure are present in the cytoplasmic matrix (original magnification × 23,000; current magnification × 13,800).

Similar abnormal large granular lymphocytes (LGL) have been described by Chan et al[11] and Semenzato et al[12] in patients with expansions of a unique population of lymphocytes consistently associated with neutropenia, a relatively favorable clinical course, and a T-suppressor/cytotoxic cell phenotype. Although recently Loughran et al[13] described three patients in whom he was able to define the leukemic nature of the LGL, the controversy regarding its malignant or benign nature remains unresolved.[14] Grossi et al[15] analyzed the granules of these LGL by enzyme cytochemistry and electron microscopy. Acid phosphatase and α-naphthyl acetate of the monocytic type were detected in the granules. The granules were also identified as primary lysosomes but were not involved in the process of phagocytosis.

Crystalline cytoplasmic inclusions in mature lymphocytes have also been observed in lymphoproliferative disorders.[16-20] In one of the cases reported by Laszlo et al,[16] the inclusions proved to be accumulations of glycoprotein and represented a stage in the evolution of Russell bodies similar to those seen in plasma cells. In two other cases with CLL reported by Clark et al,[17] the inclusions were crystalline structures that stained for specific immunoglobulins by immunofluorescence. The published data indicate that crystallization of intracellular material appearing as globular, tubular, fibrillar, or rod-shaped inclusions within peripheral blood lymphocytes in lymphoproliferative disorders is not an uncommon event. When present, the crystals represent aggregates of γ, λ, μ, or κ chains that in some cases have been antigenically identical to the membrane-bound immunoglobulin present in the cell surface.[17,21]

The classic distinctive giant granulations seen in the granulocytes of the Chédiak-Higashi syndrome can also be seen in the lymphocytes of patients with this disorder. The physical similarity of the giant granules present in this syndrome to the normal granulation present in normal granulocytes has been documented by Bessis et al[22] in light and electron microscopic studies. The cytochemical profile of these giant granules is also compatible with normal granulocytic particles whose histochemical and biochemical evaluation have proven them to be lysosomes.[23-24] In addition, White,[25] using techniques of ultrastructural histochemistry, corroborated that the Chédiak-Higashi giant granules were abnormal lysosomes.

Other types of giant inclusions were also observed by

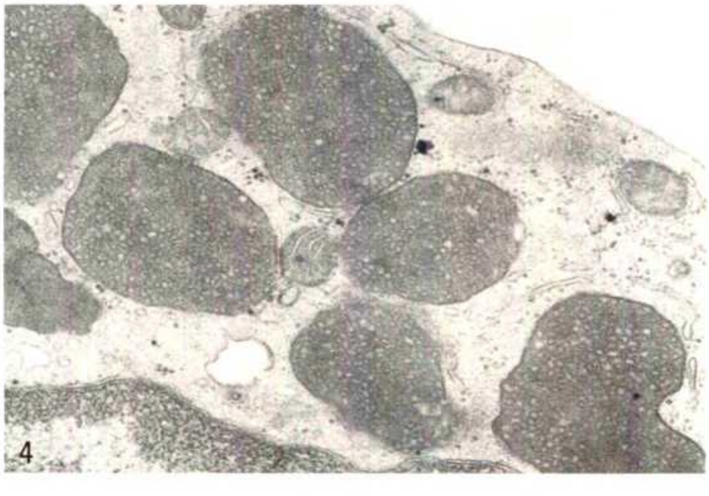

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Fig 4. The cytoplasm of another lymphoblast from the patient is filled with membrane-enclosed inclusions. Vesicular organization of the internal matrix of the large organelles is again apparent (original magnification × 43,000; current magnification × 25,800).

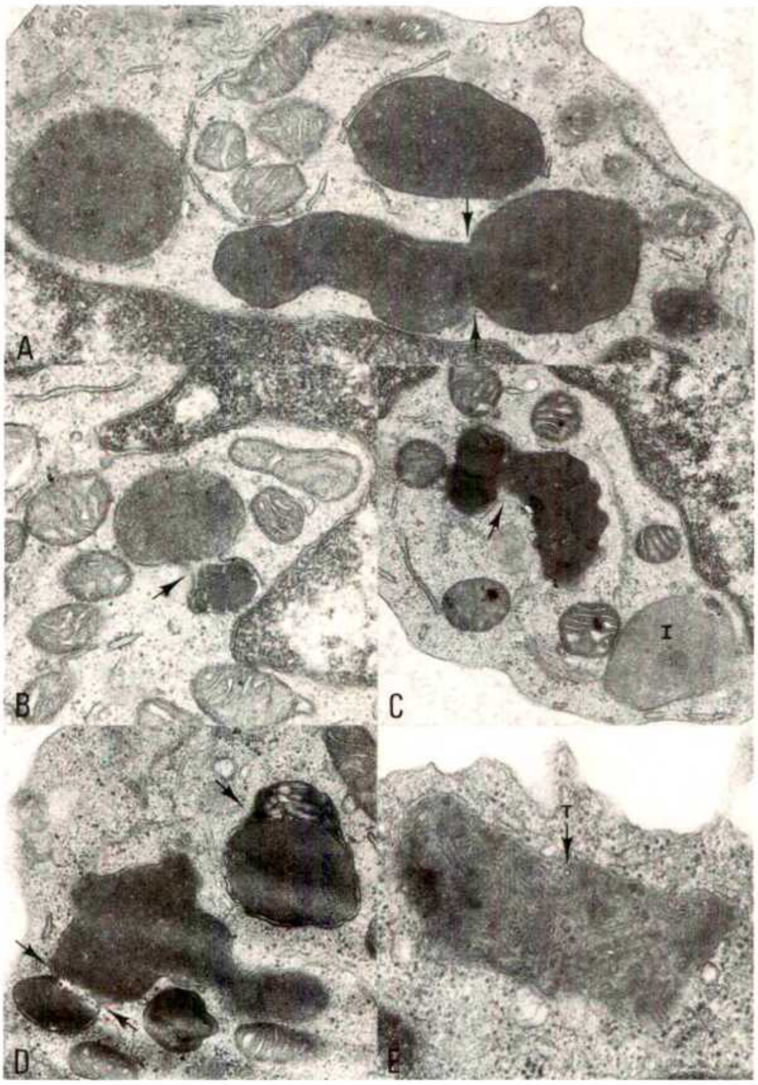

Fig 5. (A) The large size of the vesicular organelles may be due to progressive fusion. Note a line (↑) of apparent fusion in this example (original magnification × 30,000; current magnification × 19,500). (B) In this example a large organelle (↑) with vesicular substructure appears to have fused with an organelle containing a more electron-dense matrix (original magnification × 37,000; current magnification × 24,050). (C) A large organelle (↑) in this lymphoblast appears to have resulted from fusion of many small organelles with an electron-dense matrix. A more typical inclusions (I) with a vesicular internal organization is apparent at the lower right (original magnification × 24,000; current magnification × 15,600). (D) Another lymphoblast contains two large, vesicular organelles showing signs of fusion (↑) with other cytoplasmic organelles (original magnification × 18,000; current magnification × 11,700). (E) Giant tubular inclusion in lymphocyte from patient with the Chédiak-Higashi syndrome. The packed tubules (T) with hollow cores bare a slight resemblance to but differ from vesicular substructure in the unusual organelles in lymphoblasts from our patient (original magnification × 40,000; current magnification × 26,000).

White[26] in mature lymphocytes of patients with the Chédiak-Higashi syndrome. The giant size of the inclusions apparently was the result of fusion of smaller organelles. Since inclusions were negative for acid phosphatase, White postulated the possibility that giant lysosomes could have fused with nonlysosomal organelles to produce compound inclusions. Similar inclusions had previously been reported by Hoving et al[27] in 1968 in a patient with chronic rheumatoid arthritis and leukopenia. Subsequently, Huhn[28] reported similar organelles in the peripheral blood lymphocytes of healthy individuals. The electron microscopic studies of these giant granules revealed a characteristic tubular substructure completely different from the vesicular structure characteristic of the giant granules in our case.

The atypical lymphocytes observed in infectious mononucleosis may also contain large cytoplasmic azurophilic granules that on electron microscopy consist of parallel arrays of microtubulelike structures and have been referred to as parallel tubular arrays (PTA) in the literature.[29] These structures had been identified by Huhn,[28], Hovig et al,[27] and White[26] in their studies of the large granules observed in cases of rheumatoid arthritis, in normal individuals, and in the lymphocytes of cases of the Chédiak-Higashi syndrome, respectively. The PTA appear to represent normal organelles occuring in lymphocytes at a particular stage of their life cycle.

To our knowledge, coarse cytoplasmic granulation or the presence of inclusions in lymphoblasts has never been previously reported, with the possible exception of Rosen et al[30] who reported the only instance we have been able to find of unusual cytoplasmic inclusions in the blasts of a patient with acute leukemia. Unfortunately, these authors could not determine the type of leukemia in their case since the blasts had morphologic features of both lymphocytic and granulocytic tissue; neither TdT or immunologic studies were performed. In the Wright's-Giemsa–stained films, the inclusions appeared as oval, pale blue, yellow-tinged organelles about 2 to 4 μm in diameter. As in our case, the inclusions gave negative results with all the tested stains, including PAS, Sudan black B, α-naphthyl acetate, oil red 0, and toluidine blue. These authors concluded that the granules described were not specific for any particular type of leukemia. The observed inclusions appear to be similar to the ones reported by Coppola and O'Connor[31] in a patient with a myeloproliferative disorder. In both instances the inclusions were morphologically different from the ones observed in our case when studied with the light microscope in Wright's-Giemsa stained preparations.

In summary, we have described a well-documented patient with ALL in whom blast cells containing large cytoplasmic granules were present. The Wright's-Giemsa staining characteristics as well as the electron microscopic features of the granules were distinctly different from other lymphocyte granules and inclusions thus far reported in the literature. Although in the electron microscopic studies the granules resembled the granules described by White[26] in the mature lymphocytes of the Chédiak-Higashi syndrome, the vesicular ultrastructure of the granules present in our case is different and, to our knowledge, hitherto undescribed. The rare occurrence of these granulations in lymphoblasts should be taken in consideration when performing the morphologic study of cases of acute leukemia. Morphologists should recognize this rare entity to avoid confusion in the proper classification of blast forms. On the other hand, the important role of cytochemistry and membrane marker studies in the characterization of blast cells is dramatized by the possible presence, albeit rare, of nonspecific abnormal granulation in lymphoblasts.

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Dibutyryl Cyclic Adenosine Monophosphate Reduces Expression of *c-myc* During HL-60 Differentiation

By S. Spence McCachren Jr, Joni Nichols, Russel E. Kaufman, and James E. Niedel

The human promyelocytic leukemia cell line HL-60 is induced to differentiate along a myelocytic pathway by dibutyryl cyclic adenosine monophosphate (dbcAMP). Other cAMP analogs are ineffective as inducing agents. The effect of these compounds on expression of *c-myc* was investigated using a DNA probe for *c-myc* to detect RNA transcripts. The dose response and time to commitment for reduction in *c-myc* expression with dbcAMP was similar to the findings for phenotypic changes. Bromo-cyclic AMP and butyrate alone caused no changes in *c-myc* expression in 24 hours, but demonstrated dramatic synergism together, suggesting that butyrate contributes in part to the effects of dbcAMP. Evidence for mechanisms of action of cAMP other than activation of the cAMP-dependent protein kinase is reviewed.

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THE HUMAN PROMYELOCYTIC leukemia cell line HL-60 is a useful model for the study of cellular differentiation. These cells grow as a relatively homogeneous population of promyelocytes, but can be induced by a variety of agents to differentiate along either a monocytic or a myelocytic pathway.[1-16] Concomitant with the development of mature phenotypic characteristics are both the loss of proliferative potential and a dramatic reduction in messenger RNA transcribed from the cellular oncogene *c-myc*.[17-22] The *c-myc* gene is highly amplified in HL-60 cells, and *myc* mRNA is present at high levels.[23,24] The *myc* gene product is felt to play an important role in control of cell growth, but it has been difficult to delineate the dependence of its expression on proliferation as opposed to differentiation of cells. A recent study in HL-60 cells suggests that a decline in *c-myc* expression depends on cell differentiation, not merely on cessation of proliferation.[25] This is also suggested by the work of Koller et al who demonstrated that apparent in vivo differentiation of cells from blast-phase chronic granulocytic leukemia by mithramycin was accompanied by reduction in expression of *c-myc*.[26]

Phorbol diesters are among the most potent and efficacious inducers of monocytic differentiation of HL-60 cells and rapidly cause a decline in the expression of *c-myc*, preceding the development of phenotypic differentiation.[9,10,19,20] The action of phorbol diesters is felt to be due to the direct activation of protein kinase C, with subsequent events dependent on phosphorylation of particular proteins.[27]

N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphate (dbcAMP)-induced differentiation of HL-60 cells shares several characteristics with phorbol diester induction. The effect of dbcAMP is rapid, with phenotypic changes apparent at 24 hours and essentially 100% of the cells displaying the mature phenotype.[8,28] This compound is also believed to act by direct activation of a protein kinase, the cAMP-dependent protein kinase. However, its actual mechanism of induction of differentiation in HL-60 cells has been uncertain because a very effective activator of the kinase, 8-bromo-cAMP (BrcAMP), is an ineffective inducer of differentiation.[8,28,29]

In this study, we extend previous observations on phenotypic changes during dbcAMP-induced differentiation to include changes in gene expression. An unusual pattern of variation of *c-myc* expression during cell differentiation is described. Finally, synergism between butyrate and BrcAMP is demonstrated.

MATERIALS AND METHODS

Cells. HL-60 cells were obtained from American Type Culture Collection (Rockville, Md) and were maintained in suspension culture in RPMI 1640 (GIBCO, Grand Island, NY) with 2 mmol/L glutamine, 10 mmol/L HEPES, penicillin (50 U/mL), and streptomycin (50 μg/mL) in 10% heat-inactivated fetal bovine serum. Mortality as assessed by trypan blue exclusion was consistently less than 4%. All inducing agents were stored dessicated at −20 °C and dissolved in the culture medium immediately prior to use. For induction experiments, cells were cultured at concentrations of 0.5 to 1.0×10^6 cells/mL. Inducing agents and reagent-grade chemicals were purchased from Sigma Chemical Co (St Louis) unless otherwise specified.

Assessment of differentiation. Cellular proliferation, chemotactic peptide receptor expression, morphology, and nitroblue tetrazolium (NBT) reduction were determined as previously described.[8,28]

Preparation of total cellular RNA. Total cellular RNA was extracted from 5 to 10×10^8 cells with guanidine hydrochloride (Bethesda Research Laboratories [BRL], Bethesda, Md) as described.[30,31] Aliquots of the RNA were denatured with deionized glyoxal (BRL) by the method of Thomas,[32] electrophoresed on 1.1% agarose gels (BRL) in 10 mmol/L sodium phosphate, pH 6.5, at 45 mA, and transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) by the method of Southern.[33] Hind-III-digested λ-phage (BRL) was used as the molecular weight standard. RNA was visualized with ethidium bromide under UV light.

Cytoplasmic RNA blots. Cytoplasmic RNA was prepared and blotted as described.[34] For each preparation, 1 to 5×10^6 HL-60 cells were harvested, and then 0.5 to 10 μL of the final 100-uL volume was diluted as appropriate for blotting on nitrocellulose. Cytoblots confirmed the results of the Northern blots and were therefore used for the majority of the experiments.

Hybridization analysis of RNA blots. Our probe for *c-myc* is a plasmid containing the *Cla*I-*Eco*RI fragment of exon 3 of a *c-myc* isolated from HL-60 cells. Labeled probe was prepared by nick translation using reagents from BRL and ^{32}P-labeled dCTP (Amersham, Chicago) to a specific activity of 1 to 2×10^8 cpm/μg. Nitrocellulose filters with bound RNA were boiled five minutes in 20 mmol/L Tris, pH 7.6, then prehybridized at 42 °C for 12 hours in a solution of 50% deionized formamide, 5× SSC (1× SSC = 0.15

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mol/L NaCl/0.015 mol/L trisodium citrate), 1% sodium dodecyl sulfate (SDS), 5× Denhardt's solution (50× = 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), and 150 μg/mL denatured, sonicated salmon sperm DNA. Hybridization used the prehybridization solution plus probe at 42 °C for 24 to 36 hours. Filters were then washed in 2× SSC, 0.1% SDS (four times at room temperature for five minutes each), then 0.1× SSC, 0.1% SDS (twice at 55 °C for 15 minutes each), and then 0.1× SSC (twice at room temperature for five minutes each). Filters were dried with a heat lamp, wrapped in Saran wrap, and autoradiographed using Kodak XAR-5 film and Lightning-Plus intensifying screens (Du Pont, Wilmington, Del) at −70 °C. Quantitation of signal intensity was done using a densitometer (Helena Laboratories, Beaumont, Tex).

RESULTS

Time course of c-myc mRNA reduction by dbcAMP. HL-60 cells were treated with 500 μmol/L dbcAMP and harvested at intervals from 0 to 48 hours. The cells developed differentiated characteristics, including loss of proliferative capacity, expression of chemotactic peptide receptor, and ability to reduce NBT, consistent with previously reported data.[8,28] For each experiment, differentiation was verified at 48 hours by a positive NBT reaction (>90% cells positive) and cessation of proliferation (data not shown). Northern blot analysis of purified total cellular RNA confirmed that our probe hybridized to a single band of RNA measuring 2.7 kilobases, the correct size for the *c-myc* message (Fig 1).[17] The time course of *c-myc* mRNA regulation as assessed by Northern blot and cytoplasmic blot analysis demonstrated an initial rapid decline in expression of *c-myc* to very low levels within the first four hours of treatment. After this initial decline, two patterns of expression were seen (in a total of six experiments). In one pattern, *myc* mRNA accumulation remained low; in the other, a moderate, transient increase was seen. In spite of these differences in the pattern of *c-myc* expression, the cells were uniformly differentiated at 48 hours by the criteria of inability to proliferate and reduction of NBT. The pattern of *c-myc* expression did not correlate with initial cell density, cell passage number, or ability to respond to other inducers of differentiation (phorbol myristate acetate or dimethyl sulfoxide).

Dose response to dbcAMP. HL-60 cells were treated with varying concentrations of dbcAMP, and cytoplasmic blots were prepared at both four and 48 hours. There was a threshold for reduction in expression of *c-myc* at approximately 50 μmol/L dbcAMP, corresponding to the threshold for phenotypic differentiation (Fig 2).

Commitment to c-myc mRNA regulation. Cells were treated with 500 μmol/L dbcAMP. At the indicated times, they were washed and resuspended in fresh medium without inducer. After 24 hours in fresh medium, the cells were assayed for loss of proliferative capability and for expression of *c-myc*. Irreversible commitment to phenotypic differentiation required exposure of the HL-60 cells to dbcAMP for at least 12 hours.[8,28] Irreversible inhibition of *c-myc* expression likewise required exposure to the inducer for at least 12 hours (Fig 3).

Synergism between butyrate and cAMP. Although dbcAMP causes a rapid decline in expression of *c-myc*,

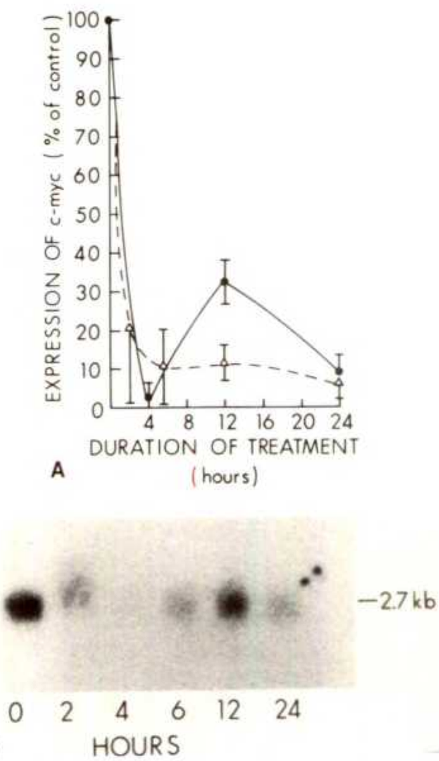

Fig. 1. Time course of *c-myc* regulation by dbcAMP. (A) Two patterns of change in *c-myc* expression were seen over time after treatment with 500 μmol/L dbcAMP. Expression was determined in a total of six experiments using a radiolabeled probe for *c-myc* hybridized to northern blots of total cellular RNA (10 μg RNA per lane) or to cytoplasmic dot blots. The average of two experiments showed an increase in expression at 12 hours (●, error bars denote the range of single determinations of duplicate experiments). The average of the other four experiments did not show such an increase (△, error bars denote SEM). (B) A representative northern blot of total cellular RNA demonstrates an initial decline in *c-myc* expression followed by a transient accumulation of message.

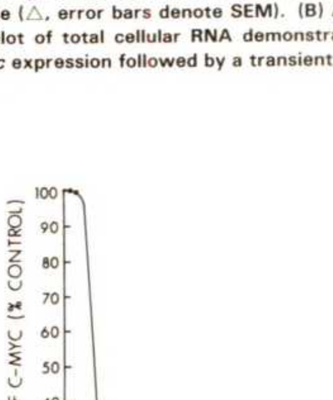

Fig 2. Concentration dependence of *c-myc* regulation by dbcAMP. The dose response of *c-myc* expression after four hours exposure of HL-60 cells to dbcAMP demonstrates a threshold effect between 25 μmol/L and 50 μmol/L dbcAMP for reduction of expression. Identical results were seen when the analysis was performed after 48 hours' exposure to dbcAMP.

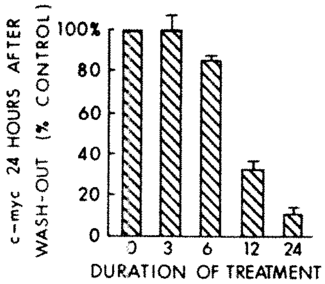

Fig 3. Regulation of *c-myc* expression as a function of duration of dbcAMP treatment. Commitment of *c-myc* regulation by dbcAMP was analyzed by treating HL-60 cells for the indicated times with 500 μmol/L dbcAMP, then washing the cells and resuspending them in fresh medium without dbcAMP. After 24 hours incubation in fresh medium, *c-myc* expression by cytoplasmic RNA blot. Error bars represent the range of single determinations in duplicate experiments.

BrcAMP, an effective activator of cAMP-dependent protein kinase, is ineffective at altering *c-myc* expression and at inducing HL-60 differentiation.[8,28] This suggested that part of the activity of dbcAMP might be due to synergy between the butyrate and cAMP portions of the molecule. We treated HL-60 cells for 24 hours with either 500 μmol/L dbcAMP, 500 μmol/L BrcAMP, 1000 μmol/L sodium butyrate, or both BrcAMP and sodium butyrate. Expression of *c-myc* was unaffected by butyrate or BrcAMP alone, but was dramatically reduced by the two agents in combination (Fig 4). Although synergy between the two agents was demonstrated, the reduction in *c-myc* expression was not quite as great as that due to dbcAMP alone.

DISCUSSION

The phenotypic differentiation of HL-60 cells in response to dbcAMP has been well characterized.[8,28] We have shown that the dbcAMP-induced reduction in *c-myc* mRNA expression demonstrates the same concentration dependence and time to commitment as does phenotypic differentiation.

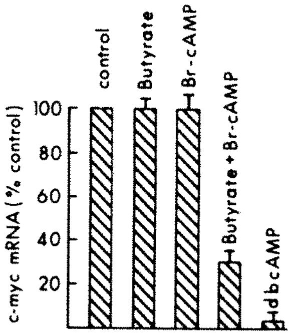

Fig 4. Synergistic effect of butyrate and BrcAMP on *c-myc* expression. HL-60 cells were treated with sodium butyrate (1000 μmol/L), BrcAMP (500 μmol/L), dbcAMP (500 μmol/L), or both the butyrate and BrcAMP for 24 hours and *c-myc* expression analyzed by cytoplasmic RNA blot. Butyrate and BrcAMP were synergistic in the reduction of *c-myc* expression, although ineffective alone. Error bars represent the range of single determinations in duplicate experiments.

Reduction of *c-myc* expression precedes phenotypic differentiation, suggesting that changes in *c-myc* may be related to the process by which dbcAMP induces differentiation. This is similar to results found with other inducers of differentiation, including dimethyl sulfoxide, retinoic acid, vitamin D_3, and phorbol myristate acetate.[17-21] The rate of reduction in *c-myc* expression is not directly related to the rate of phenotypic differentiation since even a late inducer such as dimethyl sulfoxide may dramatically decrease expression of *c-myc* within one hour.[21] The fact that all inducers of HL-60 differentiation thus far tested have reduced the expression of *c-myc* confirms the inverse relation between *c-myc* expression and differentiation. HL-60 cells induced to stop proliferating by nutrient deprivation do not show a reduction in *c-myc* expression, also suggesting that it is differentiation, rather than proliferation, to which reduction in *c-myc* expression is related.[25] In several experiments, we noted a secondary transient increase in *c-myc* expression after treatment of cells with dbcAMP. Similar results have been reported in mouse erythroleukemia cells induced to differentiate with dimethyl sulfoxide.[35] The significance of this secondary increase in expression is uncertain.

In general, agents that raise intracellular cAMP levels are effective as inducers of HL-60 differentiation in proportion to the degree to which they raise cAMP. This is true for prostaglandin E_2 plus theophylline, cholera toxin, dbcAMP, and *Bordatella* adenylate cyclase toxin.[8,22,28] The major exception is BrcAMP, an effective activator of cAMP-dependent protein kinase, which does not induce differentiation or reduce *c-myc* expression. Since BrcAMP has no effect on *c-myc* expression, we were prompted to look for synergy between butyrate and cAMP to explain the rapid effect of dbcAMP. Synergism was observed, although the reduction of *c-myc* expression was not quite as great with the two agents as with dbcAMP alone.

cAMP exerts its effect by binding to the regulatory subunits of the cAMP-dependent protein kinase holoenzyme, freeing the catalytic subunits.[36,37] Each regulatory subunit has two cAMP binding sites, and there are two major types of regulatory subunits. BrcAMP and dbcAMP (whose active form is monobutyryl cAMP) have differing affinities for the two intrachain binding sites and also differ in affinity for the two types of regulatory subunits.[38-41] These differences are most apparent in the dissociation of cAMP from the regulatory subunits.[39] As the concentration of dbcAMP or BrcAMP is increased, however, all binding sites eventually become occupied by the cAMP congener. Therefore, at high concentrations, both BrcAMP and dbcAMP are effective activators of the kinase. If kinase activation was the sole mechanism by which cAMP mediated differentiation, then similar effects would be expected from BrcAMP, dbcAMP, and the *Bordatella* adenylate cyclase toxin.

An alternative hypothesis is based on observations in bacteria in which it is known that some cAMP-binding proteins are potent regulators of gene expression and form complexes with specific DNA sequences. By analogy, induction of HL-60 cells by cAMP may be mediated in part by a cAMP-regulatory subunit complex that displays binding affinity for specific DNA sequences.[42] Indeed, there are multiple regions of homology between bovine regulatory subunit II and the bacterial cAMP-binding protein that binds DNA.[43] There are also regions of sequence homology in the 5'-flanking regions of bacterial and eukaryotic genes regulated by cAMP.[42,44] If this mechanism is involved in eukaryotic gene regulation, then the lack of differentiating activity of BrcAMP may be explained by postulating that the bulky bromine substituent prevents the BrcAMP-regulatory subunit complex from interacting with DNA.

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Subpopulations of Common Acute Lymphoblastic Leukemia Antigen-Positive Lymphoid Cells in Normal Bone Marrow Identified by Hematopoietic Differentiation Antigens

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Bone marrow samples from normal adults and children with nonhematologic malignancies not involving the marrow, acute lymphoblastic leukemia (ALL) in continued remission, and immune cytopenias were studied by two-color immunofluorescence (IF) and flow cytometry to characterize common acute lymphoblastic leukemia antigen (CALLA)-positive marrow lymphoid cells. Marrow was separated by Ficoll/Hypaque centrifugation followed by passage over a monoclonal antibody affinity column to remove myeloid cells prior to IF staining. A higher proportion of CALLA-positive cells was found in the pediatric marrows (mean, 33.7% ± 6.3% SEM) than in the adult marrows (mean, 4.5% ± 1.6% SEM). Two subpopulations of CALLA-positive cells identified by cell sorting to be of lymphoid morphology were found in both adult and pediatric marrows. A small subpopulation comprising 12.3% of the total CALLA-positive cells was characterized by a high intensity of CALLA, terminal deoxynucleotidyl transferase (TdT), and MY10 expression, but low B1, common leukocyte antigen, and peanut agglutinin receptor expression. The remainder of the CALLA-positive cells displayed low intensity of CALLA expression, positivity for the common leukocyte antigen, B1 and peanut agglutinin, and negative reaction with TdT and MY10. Both CALLA-positive subpopulations were positive for HLA-DR and the pan-B cell marker B4, but negative for cytoplasmic immunoglobulin, B2 and Leu 1. BrdU labelling studies showed that a similar proportion of cells in each subpopulation was in S phase. A slightly higher proportion of the strongly CALLA-positive cells possessed the morphologic features of high nuclear/cytoplasmic (N/C) ratio and prominent nucleoli. These studies suggest that a discrete maturation step occurs among CALLA-positive marrow lymphoid cells, resulting in the loss of TdT and MY10 expression, but gradual acquisition of the B cell marker B1 and the common leukocyte antigen. The presence of B4 antigen in nearly all CALLA-positive cells suggests that both subpopulations of normal CALLA-positive marrow cells are committed to the B cell lineage.

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THE BONE MARROW is the presumed site of origin of early B cell precursors in man.[1] The malignancy that phenotypically most closely resembles early stages of B cell maturation is acute lymphoblastic leukemia (ALL), a neoplasm arising in most cases from the bone marrow. The cells of most patients with ALL express markers specific to the B cell lineage such as B4 and B1,[2-4] as well as markers characteristic of immature lymphoid cells, such as terminal deoxynucleotidyl transferase (TdT) and the common acute lymphoblastic leukemia antigen (CALLA). It has been known for some time that neither CALLA nor TdT are specific for ALL, but can be found on a small population of bone marrow cells that coexpress B cell–associated surface antigens.[5,6] These are believed to be early B cell precursors (prior to pre-B or peripheral blood B cells in maturation) since they lack both cytoplasmic and surface immunoglobulin. We have further characterized these cells using cell surface markers present on ALL cells (TdT, MY10), mature B cells (B1, B2, common leukocyte antigen), or throughout the B cell lineage (B4, HLA-DR). With two-color immunofluorescence (IF) analysis and cell sorting techniques, we are able to identify two distinct subpopulations of CALLA-positive cells in pediatric and adult bone marrow. The heterogeneity of TdT and B1 expression observed in previous studies of CALLA-positive marrow cells is largely due to the presence of these two subpopulations, which display characteristically different combinations of cell markers. A strongly CALLA-positive subpopulation displays an antigenic phenotype suggesting an earlier maturational stage than a weakly CALLA-positive subpopulation, which bears a closer resemblance to mature B cells.

MATERIALS AND METHODS

Patients. Bone marrow aspiration from the posterior iliac crest was performed on healthy adult donors after informed consent was obtained (n = 6). Samples from diagnostic bone marrow aspirates were obtained from children with the following diagnoses: immune neutropenia (one patient), immune thrombocytopenia (two), anemia (probably secondary to viral infection) (one), neuroblastoma not involving the bone marrow (two), and ALL in complete clinical remission (six). Collection of samples was performed after approval by the institutional Committee on Investigations Involving Human Subjects and in accord with an assurance filed with and approved by the DHHS.

Cell preparation. Bone marrow cells were collected in 10-mL sterile siliconized tubes containing 5 mL of RPMI 1640 tissue culture medium, 10% fetal calf serum, and 100 U of preservative-free heparin. Ficoll/Hypaque (F/H) separation of these cells was performed.[7] Cells from patients with CALLA-positive ALL were frozen in liquid nitrogen as previously described[7] for later thawing and staining with anti-CALLA monoclonal antibody.

Affinity column cell separation. Cell separation by affinity column was performed by the method of Berenson et al.[8] Monoclonal antibodies MMA and OKM1 were obtained as hybridomas from the American Type Culture Collection (Rockville, Md) and grown in pristane-treated mice for the production of ascites fluid. These antibodies bind to myeloid cells in the bone marrow[9,10] but are not present on ALL cells. A mixture of these monoclonal antibodies each at 2 mg/mL was coupled to Sepharose cyanogen bromide–activated beads (Sigma Chemical Co, St Louis) as described.[8]

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Two milliliters of prepared beads were loaded in elution buffer (degassed phosphate-buffered saline [PBS] with 2% bovine serum albumin) into a 3-ml glass syringe and cooled at 4 °C for one hour prior to loading the cells. Cells were prepared at a total number of 10 to 20 × 10^6 cells in 200 μL of elution buffer at 4 °C. The cell suspension was applied to the column and allowed to sediment into the column bed, after which 200 μL of elution buffer was added and the column allowed to incubate at 4 °C for two hours. Five to 10 ml of nonadherent cells were eluted with elution buffer at 4 °C using a peristaltic pump at a flow rate of 0.125 mL/min. Beads were prepared for reuse as described[8] and stored at 4 °C in PBS with 0.05% azide.

Phycoerythrin conjugation of monoclonal antibody. To stain cells directly for CALLA and common leukocyte antibody (CLA), a monoclonal antibody with anti–common leukocyte specificity (GAP 8.3[11]) was conjugated with phycoerythrin (PE) using the method of Oi et al.[12] Briefly, *N*-succinimidyl 3-(2-pyridylthio)-propionate (SPDP) (Pharmacia Fine Chemicals, Piscataway, NJ) was incubated with purified monoclonal antibody as described.[12] A pyridyldisulfide derivative of R-phycoerythrin (Molecular Probes, Junction City, Ore), treated with 0.5 mol/L dithiothreitol and dialyzed, was incubated in a molar ratio of 1:5 with the SPDP antibody conjugate for 12 hours at room temperature (RT). Remaining sulfhydryl groups were blocked with sodium iodoacetate. Antibody-PE conjugates were separated from free antibody and free PE by Sephacryl-300 gel filtration and hydroxyapatite column chromatography. The most active fractions were pooled for use in IF staining.

IF staining. Three different staining protocols were necessary because some monoclonal antibodies were available as PE conjugates, some as fluorescein isothiocyanate (FITC) conjugates, and others only as purified antibody. Monoclonal antibodies were obtained from Becton Dickinson Monoclonal Center (anti-CALLA FITC, Leu-1 PE, HLA-DR FITC, MY10, anti-BrdU FITC, avidin PE, FITC- and PE-conjugated irrelevant murine IgG1, and FITC-conjugated irrelevant murine IgG2), Coulter Immunology (Hialeah, Fla) (B1 FITC, B2 FITC, B4, J5 biotin, biotin-conjugated irrelevant murine IgG2), Bethesda Research Laboratories (Bethesda, Md) (anti-TdT), Southern Biotechnology (Birmingham, Ala) (isotype-specific goat antimouse IgG1/FITC), and Vector Biochemicals (Burlingame, Calif) (peanut agglutinin [PNA] FITC).

The choice of anti-CALLA reagents (anti-CALLA FITC or J5 biotin followed by avidin PE) was based on the availability of FITC or PE conjugates of the second marker to be studied. For two-color IF of CALLA *v* Leu-1, HLA-DR, or CLA, appropriate titered dilutions of anti-CALLA FITC and Leu-1 PE, HLA-DR PE, GAP 8.3 PE (anti–common leukocyte), or PE-conjugated irrelevant murine IgG1 were added to 0.5 × 10^6 cells in PBS and incubated for 30 minutes at 4 °C. The Leu-1, HLA-DR, or CLA reactivity of CALLA-positive cells was compared with the negative control (anti-CALLA FITC and PE-conjugated irrelevant murine IgG1). Cells were also stained with FITC-conjugated irrelevant murine IgG2 and PE-conjugated irrelevant murine IgG1 to serve as a negative control for the anti-CALLA FITC.

For two-color IF of CALLA *v* B1, B2, or PNA, the cells were incubated in J5 biotin for 30 minutes at 4 °C followed by three washes with PBS and incubation with avidin PE plus either B1 FITC, B2 FITC, PNA FITC, or FITC-conjugated irrelevant murine IgG1 for 30 minutes at 4 °C. The B1, B2, or PNA reactivity of J5-positive cells was compared with the negative control (J5 biotin and FITC-conjugated irrelevant murine IgG1 followed by avidin PE). Cells were also stained with biotin-conjugated irrelevant murine IgG2 and FITC-conjugated irrelevant murine IgG1 followed by avidin PE to serve as a negative control for the J5 biotin.

Two-color IF using J5 biotin (developed by avidin PE) and a second unconjugated monoclonal antibody (developed by FITC-conjugated goat antimouse Ig) required special care to avoid cross-binding of the goat antimouse reagent to both primary mouse monoclonal antibodies. Since B4 and MY10 (both IgG1 isotypes) were only available as unconjugated antibodies, an isotype-specific goat antimouse IgG1/FITC reagent was used in the second step to prevent binding of the FITC-developing reagent to the J5 (IgG2a isotype) biotin. The cells were incubated in J5 biotin and either B4, MY10, or irrelevant murine IgG1 (MOPC 21) for 30 minutes at 4 °C followed by three washes with PBS and incubation with avidin PE and isotype-specific goat antimouse IgG1/FITC for 30 minutes at 4 °C. The B4 or MY10 reactivity of J5-positive cells was compared with the negative control (J5 biotin and irrelevant murine IgG1 [MOPC 21], followed by avidin PE and isotype-specific goat antimouse IgG1/FITC). Cells were also stained with biotin-conjugated irrelevant murine IgG2 and irrelevant murine IgG1 (MOPC 21) followed by avidin PE and isotype-specific goat antimouse IgG1/FITC to serve as a negative control for the J5 biotin.

In each case, cells were washed thrice in PBS and resuspended in 300 μL of PBS at 4 °C for analysis on the flow cytometer.

Flow cytometry analysis. Samples were analyzed either fresh or after fixation with 1% paraflormaldehyde since no difference was observed in IF staining or light scatter parameters after fixation and incubation at 4 °C overnight. Stained cells were analyzed on an EPICS C flow cytometer (Coulter EPICS Division, Hialeah, Fla) using the 488-nm line of an argon laser at 500 mW power. Four parameters were measured for each cell: forward angle light scatter (FALS), logarithm of 90° light scatter (L90LS), logarithm of green fluorescence (LGFL), and logarithm of red fluorescence (LRFL). LGFL was collected through a 560-nm dichroic filter and a 525-nm bandpass filter (±20 nm), and LRFL was collected using reflected light from the 560-nm dichroic filter and passed through a 575-nm bandpass filter (±10 nm). Fluorescent microspheres (Coulter EPICS Division) were analyzed each day and the fluorescence intensity recorded. Cells were analyzed at a flow rate of 500 to 2,000 cells/s. FALS gates were set to exclude RBC, dead cells, platelets, or debris, as well as large aggregates. L90LS gates were set to exclude a distinct myeloid population with high L90LS signal. A total of 30,000 to 300,000 cells were counted per sample. A two-parameter 64 × 64-channel histogram of LGFL *v* LRFL gated on FALS and L90LS was collected as well as a 256-channel histogram of LGFL gated on FALS and L90LS.

Data analysis. Data were stored on 8-in floppy disks and analyzed using the EPICS C data analysis software. The projection analysis software was used that enables graphic analysis of the green fluorescence of cells showing a given range of red fluorescence, and vice versa. Thus, cells positive for one antibody (eg, CALLA) can be analyzed for staining with another antibody. For determination of the percentage of positive cells, a modification of the Coulter IMMUNO data analysis method was used to evaluate weakly staining cell populations. The percentage of positive cells for each antibody was calculated by comparison of the projected fluorescence histograms of specifically stained cells (stained with an anti-CALLA antibody and a second antibody) and control cells (stained with an anti-CALLA antibody and either PE-conjugated irrelevant murine IgG1, FITC-conjugated irrelevant murine IgG1, or irrelevant murine IgG1, depending on the staining protocol used) and noting the fluorescence channel in which the positive histogram diverged from the control. The control histogram was normalized to make the number of cells to the left of this cutoff in the control histogram equal to that in the specifically stained (test) histogram. The percentage of positive cells was calculated as the number of cells with a fluorescence signal greater than this cutoff minus the normalized number of cells in the control histogram with a fluorescence signal greater than the cutoff divided by the total number of cells in the test histogram. In all cases, the cutoff point was set at a point above which only 5% to 25% of the control cells were stained to avoid misinterpretation of very weak staining intensity as a positive reaction.

To determine the fluorescence intensity of a stained population, the logarithmic fluorescence channel intensity was converted to arbitrary linear units based on the determination that an increase of 6.25 log channels in the 64-channel two-parameter histogram is equivalent to doubling of the fluorescence intensity. This factor was determined using microspheres of calibrated fluorescence intensity.[13] The linear intensity thus determined was adjusted for instrument variability by comparison with the fluorescence intensity of standard fluorescent microspheres analyzed on that day.

Cell sorting. For cell sorting experiments, only fresh cells were used. Three-droplet sorting without coincidence correction at a flow rate of 300 to 500 cells/s was performed at a 32-kHz droplet formation rate. The sorting logic was based on FALS and green fluorescence alone since some interference of the stream undulation with the 90° light scatter signal was observed. Cells were sorted into a 15-mL conical centrifuge tube containing 14 mL of F/H and 0.5 mL of RPMI 1640 tissue culture medium with 10% fetal calf serum. After sorting, the supernatant layer of tissue culture medium plus cells was aspirated and cytocentrifuges prepared on a Shandon II cytocentrifuge (Sewickley, Pa).

Immunoalkalinephosphatase staining. The method of Cordell et al[14] was used. Cytocentrifuged cells were air dried overnight and fixed in formol-buffered acetone for five minutes at RT. Mouse monoclonal antibody on the cell surface from the original staining procedure (prior to sorting) was blocked by incubation with excess F(ab) fragments of goat antimouse Ig (Cooper Biomedical, Malvern, Penn) in a humid chamber for 30 minutes at RT. After washing with PBS, slides were stained with an appropriate dilution of unconjugated MY10 mouse monoclonal antibody or irrelevant mouse monoclonal IgG1 (MOPC21) for 30 minutes at RT followed by washing in PBS and a similar incubation with alkaline phosphatase-mouse antialkaline phosphatase immune complexes (Dako, Santa Barbara, Calif). After washing in PBS, slides were incubated in fast red substrate for 15 seconds and viewed under a microscope. The control preparation showed $<5\%$ positively stained cells.

BrdU incorporation. The percentage of cells in S phase was determined by in vitro BrdU incorporation followed by staining with anti-BrdU as described by Dolbeare et al.[15] Cells were incubated in RPMI 1640 tissue culture medium with 10% fetal calf serum and 10 umol/L BrdU (Sigma Chemical Co) at 37 °C for 30 minutes after F/H separation. After affinity column separation, staining, and sorting, cytocentrifuged slides were fixed in 70% ethanol for 30 minutes at RT. The slides were immersed in 0.07 N NaOH for two minutes, followed by 0.1 mol/L $Na_2B_4O_7$, pH 8.5, to neutralize the base. The slides were incubated with an appropriate dilution of FITC-conjugated anti-BrdU for 30 minutes at RT, washed in PBS, and coverslipped for viewing under an IF microscope.

TdT and cytoplasmic Ig IF. Cytocentrifuge slides were fixed in methanol for 30 minutes at 4 °C. For the TdT assay, slides were incubated with rabbit anticalf TdT for 30 minutes at RT followed by FITC-conjugated goat antirabbit Ig for 30 minutes at RT. For cytoplasmic μ-chain detection, cytocentrifuge slides were fixed in cold (4 °C) acetone for five minutes and then incubated in FITC-conjugated goat antihuman IgM (Worthington Diagnostics) for 30 minutes at RT. Slides were washed in PBS and coverslipped for viewing under an IF microscope.

RESULTS

Separation of marrow lymphocytes by affinity column. For all studies to be described, except where specifically indicated, we passed F/H-separated bone marrow cells through an affinity column containing both OKM1 and MMA monoclonal antibodies to remove myeloid cells from the bone marrow preparations. Among cells not adhering to the column we recovered an average of 77.7% ($\pm 7.5\%$ SEM, n = 8) of the marrow lymphocytes loaded on the column while achieving a 90.7% ($\pm 2.5\%$ SEM, n = 8) depletion of myeloid cells. F/H-separated marrow samples contained a mean of 17.8% ($\pm 4.7\%$ SEM, n = 8) lymphoid or undifferentiated cells, 59.8% ($\pm 5.2\%$) myeloid cells, and 21.9% ($\pm 3.6\%$) erythroid cells, whereas F/H plus affinity column-separated samples contained a mean of 41.9% ($\pm 9.9\%$ SEM, n = 8) lymphoid or undifferentiated cells, 15.5% ($\pm 4.5\%$) myeloid cells, and 42.4% ($\pm 9.7\%$) erythroid cells.

The advantages of removing myeloid cells from F/H-separated bone marrow specimens for the purpose of analyzing and sorting lymphoid subpopulations are illustrated in Fig 1. A population of cells demonstrating weak binding to both FITC- and PE-conjugated monoclonal antibodies was observed in bone marrow samples subjected to indirect IF staining after F/H separation. This staining pattern was observed regardless of whether specific or irrelevant monoclonal antibodies were used in the staining procedure. The cells did not demonstrate uptake of propidium iodide. They

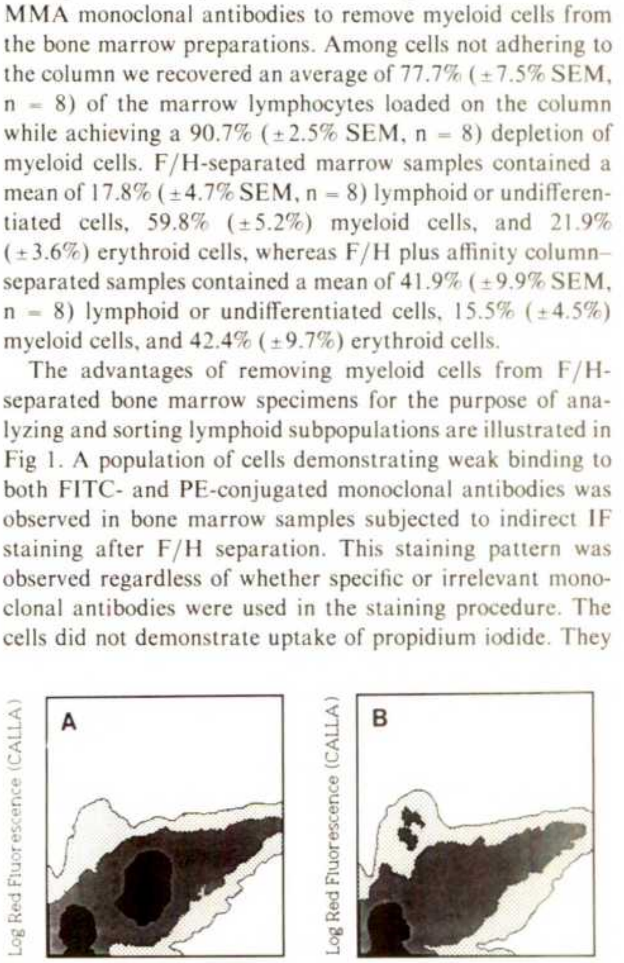

Fig 1. Effect of affinity column separation on two color IF of F/H-separated bone marrow. Samples are stained with J5 biotin plus FITC-conjugated PNA followed by avidin PE. The LGFL (PNA) is displayed on the abscissa, the LRFL (CALLA) on the ordinate. A quantity of 115,000 to 217,000 cells were counted. Contour lines indicate cells per channel of the 64 × 64-channel histograms (set at 10, 35, and 150 cells per channel for A and B and at 5, 20, and 100 cells per channel for C since this histogram contains fewer total cells). (A) Without affinity column separation or gating on 90° scatter. (B) Without affinity column separation but with gating on 90° scatter. (C) Cells not adhering to affinity column with gating on 90° scatter. Note the virtual absence of cells displaying intermediate levels of both green and red fluorescence (shown to be myeloid cells by cell sorting on three samples), with resulting clearer separation of strongly CALLA-positive, PNA-negative cells from weakly CALLA-positive, PNA-positive cells.

were demonstrated by cell sorting (n = 3) to consist of an average of 90.3% myeloid cells, mostly mature neutrophils, and 2.3% lymphocytes, with the remainder consisting of nucleated erythroid cells.

Myeloid cells and monocytes characteristically possess a high 90° light scatter signal (because of cytoplasmic granularity) on the flow cytometer.[16] However, this parameter is unable to completely separate myeloid from lymphoid cells in F/H-separated marrow, resulting in a fluorescence histogram that contains many residual nonspecifically staining myeloid cells (Fig 1B). After passage through the affinity column, the marrow is substantially depleted of these cells (Fig 1C).

Affinity column–separated (nonadherent) cells were over 95% viable by propidium iodide staining. Cells displaying CALLA positivity after column filtration were identified by sorting (n = 9) to consist of a mean proportion of 92.5% lymphocytes, indicating that nonspecifically stained myeloid cells or weakly CALLA-positive neutrophils were not being included in the analysis of CALLA-positive B lymphoid cells. This proportion was similar in both strongly and weakly CALLA-positive cells.

Frequency of CALLA-positive cells in bone marrow specimens. Bone marrow samples were studied after separation using the monoclonal antibody affinity column. The separated marrow samples contained a mean of 23.4% CALLA-positive cells (±5.3% SEM, n = 17). The histogram of CALLA FITC fluorescence of these cells suggested the presence in most marrow samples of subpopulations of cells with weak and strong CALLA intensity (Fig 2). To facilitate objective study of these cells, strongly CALLA-positive cells were defined as those cells with CALLA FITC fluroescence greater than 125% higher than the modal fluorescence of the entire CALLA-positive population. This particular cutoff was chosen because in marrow samples with a recognizably bimodal distribution of CALLA positivity (as in Fig 2) the inflection point between the peaks occurred approximately at this point. The frequency of these strongly CALLA-positive cells as a percentage of total CALLA-positive cells ranged from 0% to 21.9% (mean, 12.3% ± 1.34% SEM; n = 17). The CALLA intensity of the strongly CALLA-positive cells was 88.4% (±12.1% SEM) of the mean CALLA intensity of 16 cases of CALLA-positive ALL, as contrasted with the CALLA intensity of the remaining weakly CALLA-positive population, which was 13.7% (±1.5% SEM) of the ALL cells.

These results were compared with a larger group of pediatric marrow samples subjected to IF staining after only F/H separation to determine whether any selective loss of CALLA-positive cells occurred as a result of the affinity column separation. The F/H-separated marrow samples were stained with a mixture of anti-CALLA FITC and anti–common leukocyte phycoerythrin (CLA-PE) to distinguish the majority of the contaminating myeloid cells, which are characterized by a higher CLA intensity than the CALLA-positive lymphoid cells as verified by cell sorting (data not shown). These marrow samples contained a mean of 28.0% CALLA-positive cells (±4.7% SEM, n = 21). The percentage of CALLA-positive cells showing strong CALLA positivity as previously defined was 8.4% (±1.5% SEM) of the total CALLA-positive cells. Thus there was no evidence for significant preferential loss of strongly or weakly CALLA-positive cells in the affinity column. The percentage and CALLA intensity of CALLA-positive cells from marrows of children with immune thrombocytopenia, neutropenia, or nonhematopoietic malignancy without marrow involvement was not significantly different from those from children with ALL in complete clinical remission. The percentage of CALLA-positive cells from affinity column–separated marrows of normal adults (4.5% ± 1.6% SEM) was lower ($P < .005$) than those from marrows of children (33.7% ± 6.3% SEM) as has been previously observed.[5] The proportion of strongly CALLA-positive cells was not significantly different in the adult (13.4% ± 2.0% SEM, n = 6) and pediatric (11.6% ± 1.8% SEM, n = 11) marrows.

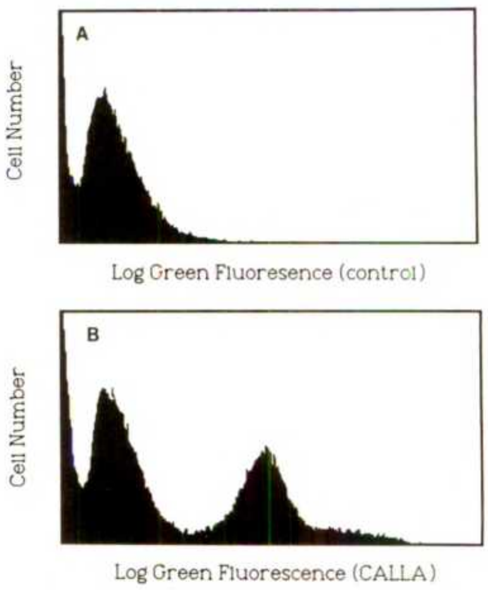

Fig 2. CALLA intensity distribution of affinity column–separated bone marrow cells. Cells were stained with FITC-conjugated irrelevant monoclonal antibody (A) or FITC-conjugated anti-CALLA (B) and analyzed on the EPICS C flow cytometer (50,000 cells counted). The abscissa is the LGFL, and the ordinate is number of cells per channel (the full scale of 256 channels of fluorescence intensity correspond to three logs of fluorescence intensity difference). The sample is gated on forward-angle light scatter to exclude RBCs and debris and 90° light scatter to exclude myeloid cells. Note the small subpopulation of cells with higher CALLA fluorescence intensity than the majority of CALLA-positive cells.

Two-color IF studies of CALLA-positive marrow cells. Two-color immunofluorescence staining of affinity column–separated bone marrow samples was performed to characterize these cells in relation to normal B cell differentiation. Antibodies reacting with surface markers potentially relevant to B cell differentiation (B4, B1, B2, CLA, HLA-DR, MY10, and PNA) were combined in a two-color assay with an anti-CALLA antibody. The choice of anti-CALLA reagents (anti-CALLA FITC [Becton Dickinson] or J5 biotin [Coulter] followed by avidin PE) was determined by the availability of FITC or PE conjugates of the B cell markers. The fluorescence intensity and relative proportion of strongly and weakly CALLA-positive cells were similar regardless of which anti-CALLA reagent was used.

Since B4 and MY10 (both IgG1 isotypes) were only available as unconjugated antibodies, an isotype-specific goat antimouse IgG1/FITC reagent was used in the second step to prevent binding of the FITC-developing reagent to the J5 (IgG2a isotype) biotin, as described in Methods. Cross-reactivity of this isotype-specific goat antimouse IgG1/FITC with mouse IgG2a (anti-CLA or anti-OKM1) was tested on normal peripheral blood lymphocytes and resulted in a fluorescence signal less than 0.5% as strong as that observed when an isotype-specific goat antimouse IgG2a/FITC reagent was used in the second step. In the bone marrow samples, the B4 or MY10 reactivity of J5-positive cells was compared with the negative control (J5 biotin and irrelevant murine IgG1 [MOPC 21] followed by avidin PE and isotype-specific goat antimouse IgG1/FITC), which always showed negligible green fluorescence.

Marrow samples stained with anti-CALLA FITC and anti-CLA PE demonstrated that the small population of strongly CALLA-positive cells showed a lower average CLA intensity (Fig 3D) than the majority of the CALLA positive population. The CLA intensity demonstrated by the weakly CALLA-positive cells was still significantly lower than that demonstrated by mature lymphocytes. The CALLA-negative, CLA-negative marrow population consisted mostly of erythroid precursors as demonstrated by cell sorting. Strongly CALLA-positive cells demonstrated binding of anti–HLA-DR to a lesser degree than the weakly CALLA-positive cells (Fig 3B), but both populations were HLA-DR–positive.

Two-color staining with anti-CALLA and MY10 showed clear-cut separation of two CALLA-positive populations, one with strong CALLA and MY10 positivity and the other with weak CALLA positivity that was negative for MY10 (Fig 3C). There was overlap in the CALLA intensity of these populations, but they were clearly separated by their reaction with MY10. These findings were confirmed by MY10 staining of sorted weakly and strongly CALLA-positive cells from a marrow sample using the immunoalkalinephosphatase technique. A small population of CALLA-negative, MY10-positive cells was also observed in some marrows. This was expected since myeloid and erythroid precursor cells are identified by this antibody.

Strongly CALLA-positive cells showed less positivity with PNA (Fig 3D) and B1 (Fig 3F) than the weakly CALLA-positive cells. Both CALLA-positive populations were B4-positive (Fig 3A), B2-negative (Fig 3G), and Leu-1–negative. The percentage of weakly and strongly CALLA-positive marrow cells positive for each antigen is shown in Table 1.

The possibility that these observed differences in antigen expression are secondary to changes in cell size alone rather than alteration in antigen density is unlikely because the forward light scatter signal, which is linearly proportional to cell volume in cells of similar structure,[17] is only 9.8% (±1.5% SEM, n = 4) higher in the strongly CALLA-positive cells. In addition, expression of some antigens was decreased, and others increased in each CALLA-positive subpopulation.

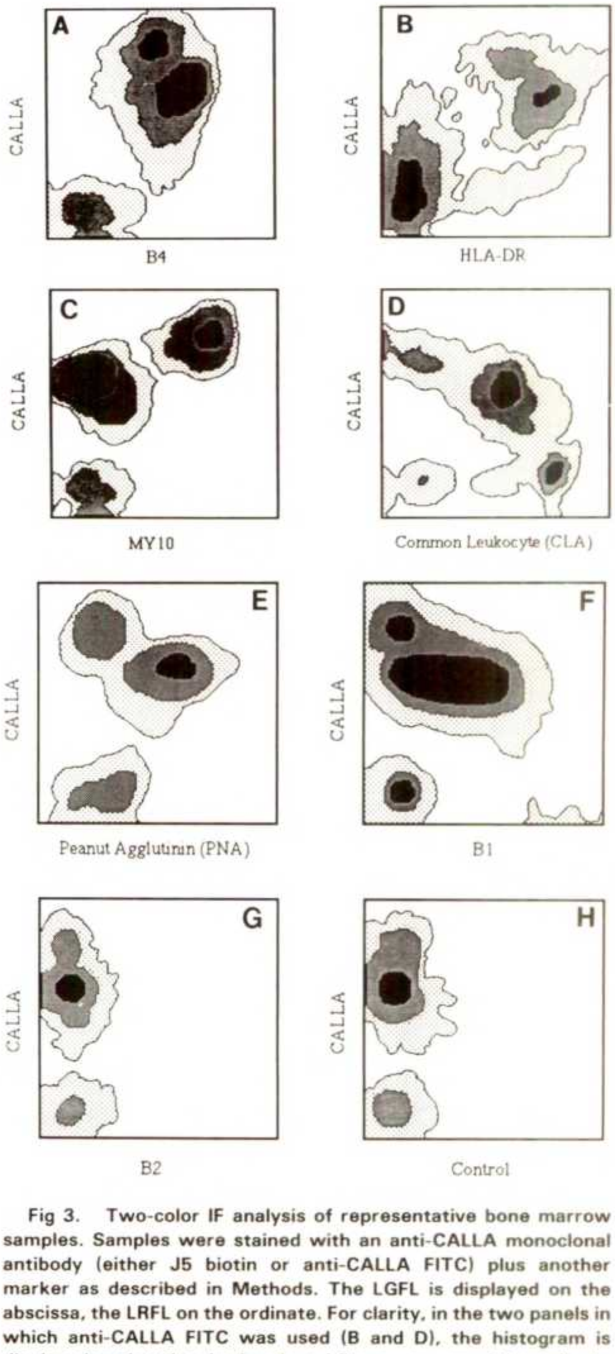

Fig 3. Two-color IF analysis of representative bone marrow samples. Samples were stained with an anti-CALLA monoclonal antibody (either J5 biotin or anti-CALLA FITC) plus another marker as described in Methods. The LGFL is displayed on the abscissa, the LRFL on the ordinate. For clarity, in the two panels in which anti-CALLA FITC was used (B and D), the histogram is displayed so that the abscissa is red fluorescence and the ordinate is green fluorescence. As a result, in all histograms CALLA intensity is represented on the ordinate and the other marker on the abscissa. All histograms except B are taken from analysis of the same marrow specimen. For each histogram 30,000 to 300,000 cells were counted. The contour lines indicate number of cells in a given channel of the 64 × 64-channel histograms. (A) CALLA *v* B4. (B) CALLA *v* HLA-DR. (C) CALLA *v* MY10. (D) CALLA *v* CLA. (E) CALLA *v* PNA. (F) CALLA *v* B1. (G) CALLA *v* B2. (H) CALLA *v* control.

As shown in Table 2, the intensity of expression of B1 and CLA in the CALLA-positive cells was significantly lower than in mature B cells. In contrast, no significant difference in intensity of expression of B4 and HLA-DR was observed between CALLA-positive marrow cells and mature B cells. Both HLA-DR and CLA expression was lower in the strongly CALLA-positive marrow cells than in the weakly

Table 1. B Cell Differentiation Markers on CALLA-Positive Marrow Cells

Monoclonal Antibody or Lectin	n	Percentage of CALLA-Positive Cells Stained for Monoclonal Antibody (±SEM) Strong CALLA	Weak CALLA	Significance Level Strong v Weak CALLA*
B4	8	88.2 (±3.4)	84.8 (±4.5)	*P* = .04
anti-HLA-DR	10	86.7 (±6.4)	91.8 (±2.6)	NS
MY10	10	88.8 (±1.5)	26.9 (±4.3)	*P* < .0001
CLA†	18	29.7 (±5.6)	62.8 (±5.9)	*P* = .006
PNA‡	10	20.0 (±3.7)	61.4 (±3.8)	*P* = .009
B1	10	20.5 (±2.3)	53.4 (±4.5)	*P* = .008
B2	6	6.8 (±2.3)	5.8 (±1.9)	NS

*Student's *t* test for paired samples.

†Anti-common leukocyte antigen (GAP 8.3).

‡Peanut agglutinin.

CALLA-positive cells. No significant difference in FALS signal was observed between either population of marrow CALLA-positive cells and the peripheral blood B cells (n = 4) used in these comparisons. The intensity of MY10 expression by the CALLA-positive marrow cells was only 42% of that displayed by the CALLA-negative MY10-positive cells (*P* = .01).

Cell sorting studies of CALLA-positive subpopulations. To obtain samples enriched in each of the CALLA-positive subpopulations, bone marrow samples were sorted on the EPICS C flow cytometer. The sort was based on forward-angle light scatter (to exclude RBCs or cell clumps) and green fluorescence. IF reanalysis of the sorted cells from five separate experiments showed an average purity of 93.6% (±2.5% SEM) of the sorted populations.

Staining of cytocentrifuged preparations of sorted cells showed that the frequency of TdT-positive cells was much higher in the strongly CALLA-positive subpopulation (Table 3). The strongly CALLA-positive subpopulation, consisting of only 11.9% of the total CALLA-positive cells, contained a mean of 57.8% (±2.9% SEM, n = 7) of all the TdT-positive cells in the whole CALLA-positive population. Both subpopulations were essentially negative for cytoplasmic immunoglobulin. Both strongly and weakly CALLA-positive cells showed a moderate proportion of cells in S phase as demonstrated by incorporation of BrdU and subsequent staining with anti-BrdU FITC. Sorted CALLA-negative cells from the affinity column–separated marrows showed less than 1% to 2% TdT- or BrdU-positive cells. The strongly CALLA-positive subpopulation contained a slightly higher proportion of cells with immature morphologic features by Wright's-Giemsa staining (high nuclear/cytoplasmic ratio, prominent nucleolus) than the other subpopulation.

DISCUSSION

CALLA-positive lymphoid cells occurring in the normal bone marrow are of interest because they are the most immature recognizable B cells in man and because they bear a phenotypic resemblance to the cells of ALL.[18] These cells are significantly more numerous in children's marrows and are found in a variety of fetal tissues.[19] The antigen has been shown to occur outside the hematopoietic system on renal epithelium[20] and breast among other tissues.

We have used two-color IF and cell sorting to examine expression on normal CALLA-positive cells of a variety of markers known to appear both early (B4[21] and TdT) and late (B1[22] and B2[23]) in B cell differentiation in addition to hematopoietic markers (CLA, MY10, and PNA) that have not been previously studied in these cells. Our results suggest

Table 2. Staining Intensity of CALLA-Positive Marrow Cells Compared With Mature B Cells

Monoclonal Antibody	n	Staining Intensity of CALLA-Positive Cells for Monoclonal Antibody Relative to Mature B Cells* Strong CALLA	Weak CALLA	Significance Levels Strong v Weak CALLA+ Cells†	All CALLA+ Cells v Mature B Cells‡
B4	9	0.58	0.65	NS	NS
anti-HLA-DR	10	0.77	1.37	*P* = .02	NS
CLA	11	0.03	0.17	*P* = .002	*P* = .002
B1§	11	0.15		—	*P* = .001

*The staining intensity of CALLA-positive marrow cells for various antibodies was compared with mature B cells present in the bone marrow samples or in separately stained peripheral blood samples. The data shown in the table are mean ratios between the indicated CALLA-positive marrow population and the mature B cells (average of at least four separate samples).

†Student's *t* test for paired samples.

‡Student's *t* test for unpaired samples. For this analysis, the staining intensity of the CALLA-positive marrow population as a whole is compared with the mature B cells.

§Only one CALLA-positive subpopulation contained substantial numbers of cells staining for B1. The data shown are based on the staining intensity of the B1-positive cells in the weakly CALLA-positive subpopulation.

Table 3. Characteristics of Sorted CALLA-Positive Marrow Cells

Marker	n	Percentage of Sorted CALLA-Positive Cells Displaying Marker (±SEM) Strong CALLA	Weak CALLA	Significance Level Strong *v* Weak CALLA*
TdT	9	80.3 (±4.5)	9.8 (±2.1)	$P < .0001$
Cytoplasmic Ig	7	2.2 (±0.7)	4.9 (±2.3)	NS
Immature morphology†	7	43.9 (±7.1)	23.1 (±5.0)	$P = .034$
BrdU incorporation‡ (percentage in S phase)	7	9.4 (±3.0)	9.7 (±2.1)	NS

*Student's *t* test for paired samples.

†Cells displaying finely granular nuclear chromatin with either prominent nucleoli or a high N/C ratio were considered to show immature nuclear morphology.

‡Cells were incubated in BrdU as described prior to staining and cell sorting, after which they were cytocentrifuged and stained with FITC-conjugated anti-BrdU.

two conclusions: (1) CALLA-positive marrow lymphoid cells are early B cell precursors, and (2) two subpopulations of CALLA-positive cells, probably representing maturation stages, can be identified in normal bone marrow.

An important requirement for quantitative IF analysis of these cells, which may be infrequent in the bone marrow, is the removal of myeloid or monocytic cells that display nonspecific binding of monoclonal antibodies. In contrast to peripheral blood, bone marrow samples contain a large number of immature and mature myeloid cells even after F/H separation. A significant proportion of these myeloid cells do not display the high 90° light scatter signal characteristic of peripheral blood neutrophils. This may be due to the presence of immature myeloid cells or activated neutrophils, which have been shown to display reduced 90° light scatter.[24] Some sort of myeloid cell depletion step is essential in two-color IF analysis of marrow specimens since the nonspecifically staining myeloid cells are likely to entirely obscure the relevant lymphoid populations, as shown in Fig 1. Substantial removal of these cells has been accomplished by immune rosetting techniques,[4] but we have found depletion by monoclonal antibody affinity column[8] to be very simple and reliable. Comparison of F/H-separated with F/H plus affinity column–separated marrow samples demonstrates that the affinity column separation does not alter the proportion of CALLA-positive cells or the inverse relationship between CALLA and CLA positivity. Cell sorting studies of CALLA-positive cells after affinity column separation demonstrates that these are indeed lymphoid cells, which is important since myeloid cells are weakly CALLA-positive.[25]

Although marrow CALLA-positive cells have been shown to possess B cell markers such as B1[22] and lack T cell–specific markers, the degree of commitment of these cells to the B lymphocyte lineage is not known. Detection of clonal immunoglobulin gene rearrangements as in ALL[26] is not applicable in a normal (polyclonal) population, and reliable culture conditions for clonal growth of these cells have not yet been developed.

The presence of B4 antigen on the surface of both strongly and weakly CALLA-positive lymphoid cells is evidence for B cell lineage of these cells since this antigen is confined to B lymphocytes.[21] The intensity of B4 expression is similar to that found on mature peripheral blood B cells. Expression of HLA-DR nonpolymorphic determinants by the CALLA-positive marrow cells is consistent with, but not specific for, B cell lineage. The significance of the different HLA-DR intensity (Table 2) of the two CALLA-positive subpopulations is unclear.

Unlike pre–B cells, both CALLA-positive subpopulations lack cytoplasmic μ-chain, consistent with previous reports.[4,5] The CALLA-positive subpopulations also lack expression of B2, which is present on over 88% of peripheral blood B cells.[27] The absence of Leu-1 staining is evidence against early T cell commitment since Leu-1 is a pan–T cell marker expressed on the most immature identifiable T cells such as T cell ALL.[28]

Correlated analysis of surface phenotype and Ig gene rearrangements of ALL cells suggests that an orderly sequential expression of B4, TdT, CALLA, B1, and B2 antigens is likely to occur during normal B cell maturation.[3,29] Previous studies have demonstrated heterogeneity of expression of TdT, B1, and B2 antigens in normal CALLA-positive cells.[4,19] The data described in this study indicate that this heterogeneity is a reflection of the presence of two subpopulations of CALLA-positive cells. The phenotypes of the two subpopulations suggest that the strongly CALLA-positive cells represent a more immature B cell subpopulation than the weakly CALLA-positive cells. This conclusion is based on differences in expression of six antigens: CALLA, TdT, MY10, CLA, B1, and PNA.

It has previously been shown[30,31] that normal CALLA-positive marrow cells display a lower mean CALLA antigen density than cells from most cases of CALLA-positive ALL. Our findings confirm these reports but demonstrate that the intensity of CALLA expression found in the strongly CALLA-positive marrow subpopulation is similar to that shown by CALLA-positive ALL cells. This feature suggests immaturity in the strongly CALLA-positive normal cells.

TdT is restricted to the lymphoid lineage and is found only in immature T or B cells and in lymphoid leukemias.[32] Hence its expression by the strong CALLA-positive subpopulation may be considered evidence of the relative immaturity of these cells as opposed to the weakly CALLA-positive marrow cells, which were characteristically TdT-negative.

The expression of MY10 by the strongly CALLA-positive cells also suggests an early state of differentiation since this

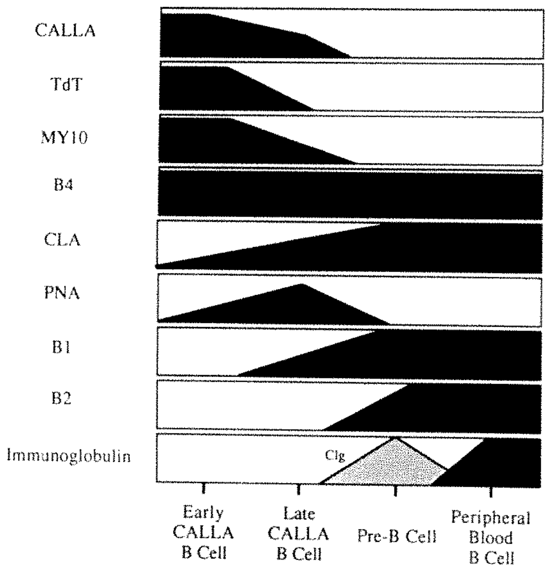

Fig 4. Graphic representation of phenotypic B cell maturation. The positivity of cells for each marker is represented by the height of the shaded area.

marker is expressed by CFU-C, BFU-E, and CFU-E and is not found on more differentiated marrow cells.[33] MY10 positivity of an immature B cell population is consistent with previously reported MY10 expression of cells from 38% of patients with CALLA-positive ALL.[33]

Comparison of strongly CALLA-positive marrow cells, weakly CALLA-positive marrow cells, and peripheral blood lymphocytes shows a progressive increase in the proportion and staining intensity of CLA-positive cells. CLA is only very weakly expressed by ALL lymphoblasts.[34] Thus the strongly CALLA-positive marrow cells more closely resemble the ALL cells, and weakly CALLA-positive cells resemble mature B cells in CLA expression. An increase in CLA positivity with maturation has been similarly observed in the T cell lineage.[34]

The majority of weakly CALLA-positive cells are B1-positive, as are nearly all peripheral blood B cells. In contrast, only a small percentage of strongly CALLA-positive marrow B cells display the B1 antigen. The CALLA-positive, B1-positive cells do show a significantly lower B1 antigen density than mature peripheral blood B cells, which may reflect gradual acquisition of this antigen during differentiation.

PNA binding is observed in certain germinal center B cells, but is not restricted to the B cell lineage. The cells of ALL have been shown to possess only weak reactivity with this lectin,[35] which was contrasted in a recent letter with the strong PNA reactivity of CALLA-positive B cells in reactive marrows.[36] The strongly CALLA-positive subpopulation, however, displays only weak binding to PNA, thus sharing a phenotypic similarity to cells from most patients with ALL that is not shared by the weakly CALLA-positive cells.

The coordinated gain and loss of cell surface and nuclear proteins of CALLA-positive bone marrow cells, shown diagrammatically in Fig 4, suggests a maturation sequence. The similarity in forward light scatter signal of the two CALLA-positive subpopulations indicates that the observed differences in antigen expression reflect alteration in antigen density rather than changes in cell size. Alternative explanations include the possibility that these subpopulations belong to different lymphoid lineages or that the phenotypic differences observed are secondary to activation and/or proliferative activity of one of the subpopulations. These explanations cannot be ruled out on the basis of our data, but the presence of B4 coupled with the absence of T cell markers in both subpopulations strongly argues for B cell lineage commitment and the similar proportion of cells in S phase in each subpopulation suggests that the antigenic differences observed are not secondary to increased entry of one population of cells into S phase.

We hypothesize that these CALLA-positive marrow subpopulations represent a maturation sequence of early B cell differentiation in man. The strongly CALLA-positive subpopulation would be expected to differentiate into the weakly CALLA-positive subpopulation and from there into pre-B and ultimately mature B cells. This hypothesis can be tested by in vitro studies with the appropriate culture conditions for demonstration of phenotypic maturation and growth of B cell colonies. A finer delineation of maturation in the B cell lineage at these early stages may aid in the understanding of normal and neoplastic B cell differentiation.

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The Malignant Cells in a Lennert's Lymphoma Are T Lymphocytes With a Mature Helper Surface Phenotype. A Multiparameter Flow Cytometric Analysis

By Kurt J. Stonesifer, Neal A. Benson, Sally E. Ryden, David F. Pawliger, and Raul C. Braylan

The flow cytometric analysis of DNA content in cells obtained from a case of Lennert's lymphoma demonstrated the presence of a discrete hypotetraploid cell population. Correlated multiparameter analysis of DNA, light scatter, and surface antigens by flow cytometry showed that the hypotetraploid cells were intermediate to large cells expressing T11, T3, and T4 antigens and lacking B1 and T8 antigens. These findings suggest that Lennert's lymphoma represents a malignant neoplasm of T-helper lymphocytes.

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IN 1968 Lennert and Mestdagh described what they believed to be a variant of Hodgkin's disease with a high content of epithelioid histiocytes and few Reed-Sternberg cells.[1] In 1976, Burke and Butler speculated that so-called Lennert's lymphoma was a distinct clinicopathologic entity and not a variant of Hodgkin's disease.[2] This idea was reinforced by Kim et al in 1978 and 1980.[3,4] It is now believed that Lennert's lymphoma is a distinct non-Hodgkin's lymphoma with a multifocal epithelioid histiocytic reaction. Using the multianalytical capabilities of flow cytometry, we studied the light scatter and antigenic expression of a hypotetraploid cell population detected by DNA measurements in a case of Lennert's lymphoma. This approach permitted a direct analysis of relative size and surface immunophenotype of the presumably neoplastic aneuploid cells in this lymphoma.

CASE REPORT

A 40-year-old white male presented in July 1984 with arthralgias and loss of strength in hand muscles over a 4-month interval, with gradual development of cervical and inguinal adenopathy, an 8-lb weight loss, and generalized weakness. An inguinal lymph node biopsy, performed elsewhere 10 months previously, was diagnosed as nonspecific hyperplasia. Because of diffuse peripheral arthritis with swelling of the metacarpophalangeal and proximal intercarpal joints, reduction of wrist motion, and loss of shoulder motion various nonsteroidal anti-inflammatory drugs were prescribed, without benefit.

On admission, he had diffuse anterior and posterior cervical and inguinal adenopathy and diffuse swelling of metacarpophalangeal and proximal intercarpal joints with restricted motion in both wrists. Laboratory findings included a normal complete blood count and electrolyte values, elevated lactic dehydrogenase level, and depressed total protein level. Antinuclear antibody and rheumatoid factor were negative, serum immunoelectrophoresis was normal, and a chest film was normal. Computerized tomography of the abdomen showed splenomegaly and prominent para-aortic and retrocaval nodes. A biopsy specimen of an inguinal lymph node the day after admission was diagnosed as malignant lymphoma with a high content of epithelioid histiocytes (Lennert's lymphoma). Lymphoma was also present in a bone marrow biopsy specimen. For the next 8 months he received 11 courses of combination chemotherapy and prednisone, and currently he has no peripheral adenopathy but does have residual lymphoma in the bone marrow.

MATERIALS AND METHODS

The first inguinal lymph node (Oct 1983) was fixed in formalin, and routine hematoxylin- and eosin-stained sections were made. The second inguinal lymph node biopsy specimen, taken in July 1984, was divided into two parts. One part was fixed in formalin, and routine hematoxylin- and eosin-stained sections were made. The other portion was minced, and the resulting monodispersed cell suspension was centrifuged through Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Md) to obtain viable mononuclear cells. An aliquot of cells was smeared onto a slide and stained with Giemsa stain for cytologic examination. Other aliquots were exposed to OKT11, OKT4, OKT8, OKT3 (Ortho Pharmaceutical Corp, Raritan, NJ), and B1 (Coulter Immunology, Hialeah, Fla) monoclonal antibodies followed by fluorescein isothiocyanate (FITC)-conjugated sheep antimouse antibody (Cappel Laboratories, Cochranville, Pa) and to FITC-conjugated goat antihuman IgM, IgG, κ and λ immunoglobulin IgG antibodies (Kallestad Laboratories, Inc, Austin, Tex). Normal mouse IgG and FITC-labeled goat IgG were used as controls. Other portions of the cell suspension were prepared for single-parameter DNA analysis,[5] and for correlated multiparameter analysis of DNA, surface antigens, and forward-angle light scatter

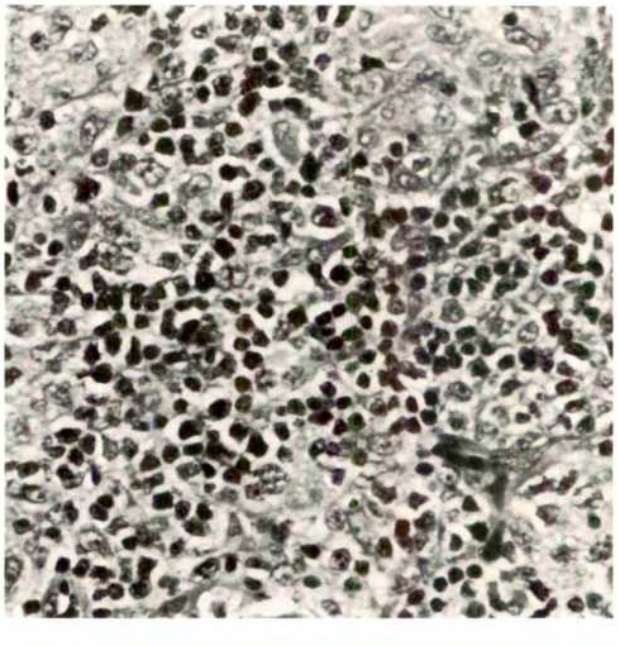

Fig 1. Inguinal lymph node biopsy, July 1984, showing features of Lennert's lymphoma. There are large numbers of epithelioid histiocytes with a background of small, intermediate, and large lymphocytes with atypical nuclei.

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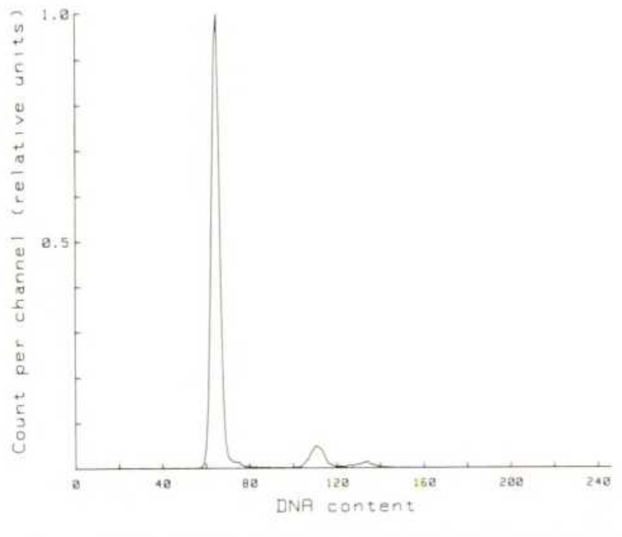

Fig 2. DNA histogram produced from flow cytometric analysis of the inguinal lymph node cells. Increasing relative DNA content per cell, indicated by the flow cytometer's channel number (x axis), is plotted v the relative number of cells in each channel (y axis). Normal G1-phase cells produced the large peak at channel 65. Normal G2-phase and mitotic cells produced the small peak at channel 130. In between, a peak of abnormal cells with a hypotetraploid amount of DNA is seen (channel 110). This abnormal population represents 7% of the total cells analyzed.

as previously described.[6] Briefly, cells labeled with FITC-conjugated antibodies were fixed with 50% ethanol, treated with RNase, and stained with propidium iodide, a red fluorescent dye that intercalates into double-stranded nucleic acids. The labeled cell suspensions were analyzed using a modified FACS II flow cytometer (Becton Dickinson, Mountain View, Calif).[6]

RESULTS

Light microscopy. Sections of the lymph nodes biopsied in Oct 1983 and July 1984 demonstrated the features of Lennert's lymphoma[2] (Fig 1). The nodes contained large numbers of epithelioid histiocytes with a background of lymphoid cells. The epithelioid histiocytes had a diffuse arrangement and also formed several discrete cell clusters. The lymphoid component consisted of small, intermediate, and large lymphocytes with irregular, cleaved nuclei. Scattered atypical mononuclear cells were present that resembled Reed-Sternberg variants with large vesicular nuclei and prominent nucleoli. The Giemsa-stained smear of the cell suspension showed predominantly lymphocytes of varying size, occasional atypical large lymphocytes, and less than 1% epithelioid histiocytes.

Flow cytometry. Analysis of surface antigens on cells from the July 1984 lymph node demonstrated the following distribution: B1, 55%; IgG, 4%; IgM, 49%; κ, 31%; λ, 22%; OKT11, 50%; OKT4, 36%; OKT8, 8%; and OKT3, 35%. DNA analysis showed that the majority of these cells had a diploid DNA content. However, a relatively small number of cells (7% of all cells analyzed) formed a discrete peak located in a hypotetraploid position in relation to the diploid cells (Fig 2). By using correlated analysis of surface antigens and DNA this discrete population of hypotetraploid cells was shown to bear T11, T3, and T4 antigens (Fig 3). T8 and B1 antigens were absent on these cells (Fig 4). The diploid cells, most likely nonneoplastic, represented a mixture of cells expressing B1 (59%), T11 (32%), T3 (28%), T4 (27%), and T8 (4%) antigens. An insufficient number of cells precluded the analysis of other antigens. Forward-angle light scatter analysis, which is related to cell size, showed the hypotetraploid cells to be of intermediate-to-large size when compared with the rest of the cell population (Fig 5).

DISCUSSION

Reports of Lennert's lymphoma progressing to a malignant lymphoma of the large cell, "histiocytic" type have been described.[7-9] Klein et al in two cases demonstrated that the histiocytic lymphoma was cytoplasmic immunoglobulin-negative but was unable to perform T cell marker studies because of a lack of unfixed tissue.[8] Miller et al described a case of histiocytic lymphoma with immunoglobulin production presumably originating from Lennert's lymphoma.[9] One

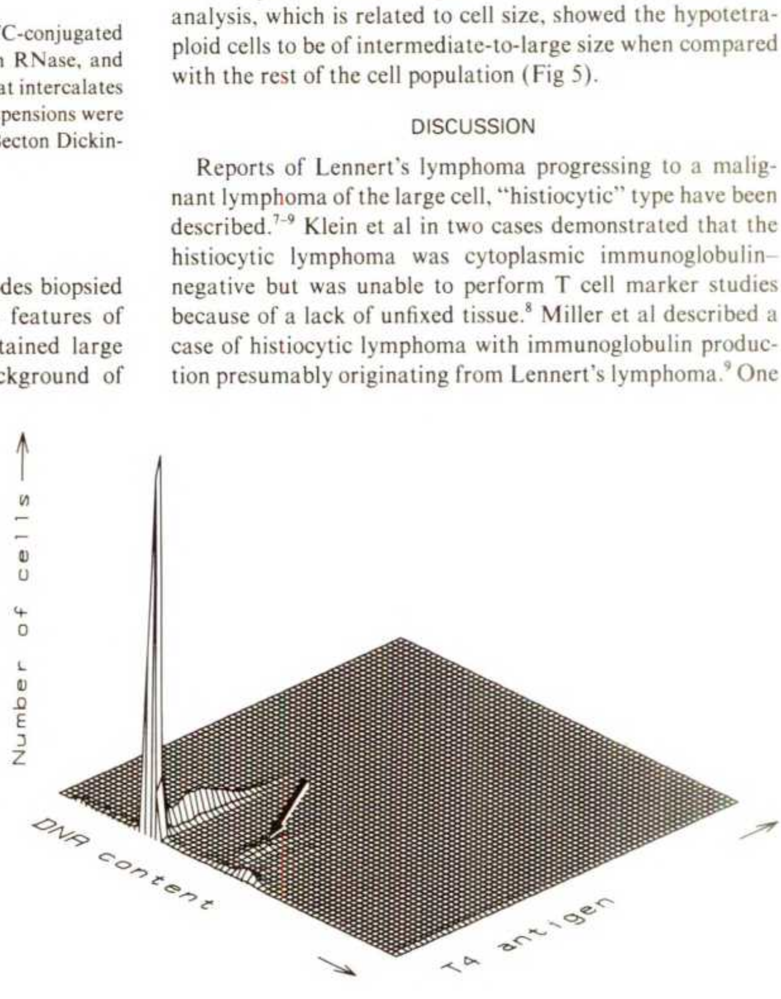

Fig 3. Correlated two-parameter histogram produced from flow cytometric analysis of the inguinal lymph node cells. One axis represents increasing relative DNA content per cell, whereas the other represents increasing expression of the T4 antigen. The number of cells is plotted vertically. The hypotetraploid cell population bearing T4 antigen is marked by the arrow.

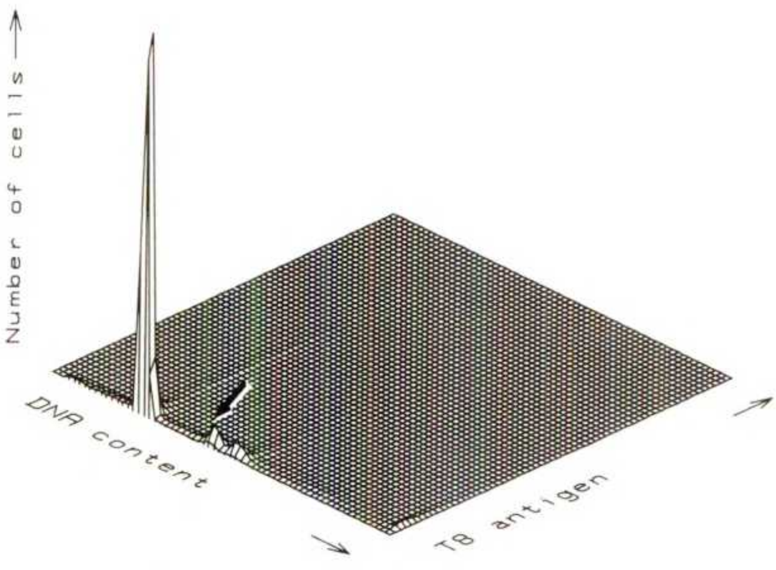

Fig 4. Correlated two-parameter histogram produced from flow cytometric analysis of the inguinal lymph node cells. This plot is similar to that in Fig 3 except that T8 antigen expression is plotted *v* increasing relative DNA content per cell. The hypotetraploid cell population is lacking T8 antigen and is marked by the arrow.

of four cases studied by Bedetti and Ollapally also demonstrated cytoplasmic staining for immunoglobulin.[10]

A number of investigators have suggested that Lennert's lymphoma is a neoplasm of T cell origin.[7,10-17] Lukes and Collins classified it as a T cell neoplasm in 1977.[17] Palutke et al and Borowitz et al found E rosetting abnormal lymphocytes in cell suspensions prepared from Lennert's lymphoma.[13,15] Bogomoletz and co-workers described a case in which OKT4 and OKT3 antibodies labeled 73% and 88% of the cells respectively.[12] Knowles and Halper demonstrated OKT3+, T10+ cells and a small number of OKT5+ and OKT8+ cells in a case of Lennert's lymphoma.[14] None of these studies, however, demonstrated conclusively the neoplastic nature of these T cells.

Some authors have even stated that Lennert's lymphoma may be a reactive condition.[8] The hypotetraploid cells detected in our sample of Lennert's lymphoma strongly suggested the presence of neoplastic cells. Ploidy abnormalities are indicative of neoplasia and hypotetraploid populations have never been observed in nonneoplastic conditions.[6,18,19] Simultaneous measurement of DNA content and light scatter showed that the abnormal cells were of intermediate-to-large size when compared with the rest of the lymphoid population obtained. Correlated analysis of DNA content and surface antigens demonstrated that the hypotetraploid cells shared surface properties with the so-called helper/inducer T lymphocytes, thus resembling other lymphomas such as cutaneous T cell lymphomas,[20] human T cell leukemia virus 1–associated leukemia-lymphomas[21] and some peripheral T cell lymphomas.[22]

The results of our analysis indicate that only a small percentage of the cells obtained in our final cell suspension had ploidy abnormalities. The remaining cells were presumably nonneoplastic since they were diploid elements expressing different surface antigens. More than half of the cells bore B cell antigens with a normal ratio of κ/λ immunoglobu-

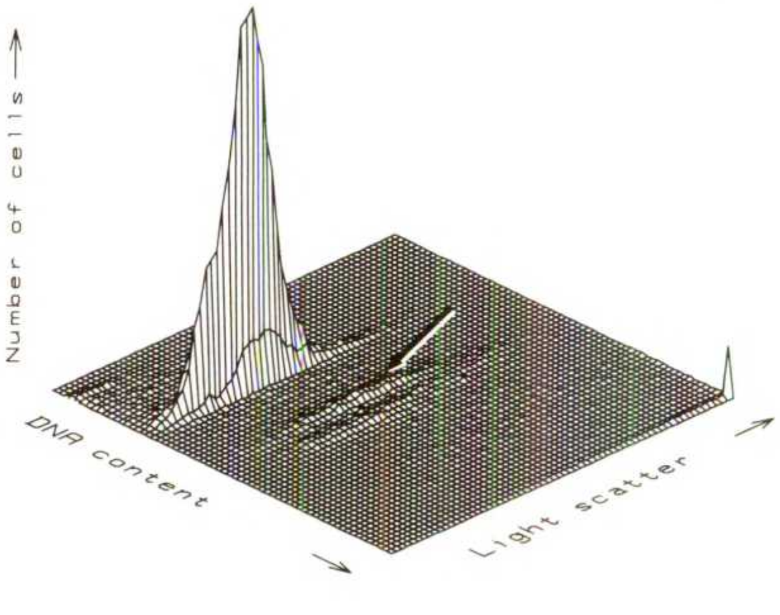

Fig 5. Correlated two-parameter histogram produced from flow cytometric analysis of the inguinal lymph node cells. This plot is similar to Figs 3 and 4 except that increasing forward-angle light scatter, an indicator of cell size, is plotted *v* increasing relative DNA content per cell. The hypotetraploid cell population, marked by the arrow, produces an intermediate-to-large amount of light scatter when compared with the total cell population.

lin-bearing cells. Also, T4 and T8 antigen–bearing cells were present in proportions that are not different than those observed in reactive processes. Although we cannot exclude the possibility that in our case the neoplastic cells, like the epithelioid histiocytes, were preferentially lost during the cell suspension preparation, the morphologic changes suggested that normal elements outnumbered neoplastic cells. Large numbers of normal-appearing cells are usually recognized morphologically in conjunction with malignant cells in Hodgkin's disease. Also, numerous apparently normal T cells are often present in B cell neoplasms.[23,24] It is likely that normal elements may also be present in T cell neoplasms, but this has not been well documented. Neoplastic T cells may produce factor(s) responsible for an active recruitment of normal cells at tumor sites. It is possible that normal lymphocytes immunoregulate the growth and/or differentiation of neoplastic cells as may happen in B cell lymphomas.[25] Regardless of the mechanism responsible for their presence, the significance of large numbers of nonneoplastic cells admixed with relatively fewer tumor cells in these lymphomas is presently unknown.

Our findings do not permit us to make a general statement about the nature of the neoplastic cells in other cases of Lennert's lymphoma. Nevertheless, this study demonstrates the power of correlated multiparameter flow cytometric analysis which can identify and characterize neoplastic cells in a manner that cannot be easily attained by other techniques. This is of particular importance when, as in our case, neoplastic elements represent only a small percentage of the cells present in a tumor. The precise identification of tumor-associated surface antigens should allow a better delineation of the cell of origin of Lennert's lymphoma and may contribute to improve its therapy.

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Antibodies to Shared Idiotypes as Agents for Analysis and Therapy for Human B Cell Tumors

By F.K. Stevenson, M. Wrightham, M.J. Glennie, D.B. Jones, A.R. Cattan, T. Feizi, T.J. Hamblin, and G.T. Stevenson

Monoclonal anti-idiotypic antibodies generated against idiotypic immunoglobulin (Ig) of neoplastic B lymphocytes can be selected from growing hybridoma clones by their ability to recognize idiotypic but not normal IgM. This group of antibodies can be subdivided into those that bind to the target tumor cells in the presence of normal human serum ($\sim$85% of the clones) and those in which binding is inhibited by serum ($\sim$15%). The former appear to be specific for private idiotypic determinants whereas the latter recognize cross-reacting idiotypic determinants. Such cross-reactivity is reflected both in recognition of a small percentage of normal Ig and also in binding to other lymphomas. The anti-idiotypes specific for private determinants can be used for therapy, with only idiotypic Ig secreted by tumor cells able to block its access to cells. The cross-reacting anti-idiotypes will face in addition the barrier of the proportion of normal Ig with which it reacts. The attraction of using a single monoclonal reagent for more than one patient has led us to develop an assay that measures the level of such blocking and to propose that those recognizing $<30\ \mu g/mL$ of normal Ig could be placed in a panel for possible therapy for several patients; less restriction need apply to antibodies for monitoring tumor progress. The assay is described, and examples of such antibodies raised against lymphoma cells from two patients are given together with comparisons with them of anti-idiotypes specific for private determinants.

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THE POSSIBILITY of using anti-idiotypic antibodies in the therapy of human B cell lymphoma has been investigated,[1] and there has been one apparent success.[2] However, there are problems remaining, such as the ability of the tumor cells to endocytose surface idiotype plus attached antibody and thereby escape from antibody attack.[3] There is also the problem of the presence of small amounts of secreted idiotype in the plasma that can block antibody and prevent attachment to the cells.[4] A recent clinical trial of anti-idiotypic antibody therapy has been reported and demonstrates the limits of the approach using simple bivalent mouse monoclonal antibody.[5] The use of univalent antibody consisting of Fab'γ exmonoclonal antibody linked chemically to host IgG allows binding to target cells but prevents endocytosis.[6] Such a molecule can invoke both complement-mediated lysis and antibody-dependent cellular cytotoxicity and has reduced immunogenicity[6] compared with whole mouse antibody.[5] Preliminary tests in vivo have shown good survival of the molecule with a half-life dictated by the attached host IgG,[6] and therapeutic trials are in progress.

However, even if this modified approach is successful, it will still be necessary to raise individual monoclonal anti-idiotype for each patient since the chance of two separate tumors having the same set of idiotypic determinants is extremely low if the incidence of such tumors reflects the full B cell repertoire. Idiotypic determinants cover a wide range of antigenicity from the private to the semipublic, and presumably the complementarity-determining regions (CDRs) involved in binding to antigen would give rise to the most private determinants. It might be expected that antibodies of similar specificities would show similar idiotypic determinants that cross-react, and this has been shown, eg, for rheumatoid factors[7] and for cold agglutinins.[8,9] However, other cross-reactions might occur outside the antigen-binding site because of the presence of less private determinants,[10] and this suggests the tempting possibility of using such an antibody to monitor and perhaps treat more than one patient. However this approach encounters the problem that a determinant semipublic as regards its occurrence on lymphocytic surfaces is likely also to be semipublic in its occurrence on extracellular Ig. Its presence there would presumably block access of antibody to target cells in the same way as tumor-derived idiotypic Ig is able to do, and therefore an additional barrier would be present. This report describes an assay that will give some measure of the degree of cross-reactivity of such antibodies and includes an estimate of the levels of blocking anticipated. If an arbitrary limit of, eg, $<30\ \mu g/mL$ of blocking Ig is imposed, it might be possible to create a panel of anti-idiotypes that can be monitored on frozen sections of lymph nodes of a wide range of patients with a view to use as monitoring and perhaps therapeutic agents. This report is concerned with analysis of typical monoclonal anti-idiotypes reacting with both private and cross-reacting determinants against the lymphoma cells of two patients.

MATERIALS AND METHODS

Patients and cell preparations. Two male patients, S.A. and N.L., aged 66 and 56 years respectively, both presented with follicular lymphoma of low-grade histology. S.A. had fairly widespread tumor present in lymph nodes and a WBC count of 7×10^9/L, approximately 50% of which were neoplastic B cells. N.L. had skin and spleen involvement and a WBC count of 10×10^9/L consisting of 90% neoplastic B cells. Tumor cells were obtained from an excised inguinal lymph node from S.A. and from the blood of N.L. by described procedures,[4] and examination of surface Ig showed that cells from both patients expressed IgMλ with a trace of IgD.

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Preparation of idiotypic IgM. Idiotypic IgM as a source of antigen for raising anti-idiotypic antibody was rescued from tumor cells by fusion with the mouse myeloma line NS-1 (P3-NS-1/1 Ag 4.1) (patient S.A.) or 653 (P3X 63 Ag 8-653) (patient N.L.).[11] After fusion, cells were distributed into a 96-well microculture plate at ~2×10^5 per well on a feeder layer of irradiated mouse thymocytes. Colonies producing human IgMλ were cloned at regular intervals in HT medium (HT = 10^{-4} mol/L hypoxanthine, 1.6×10^{-5} mol/L thymidine) by limiting dilution to guard against spontaneous loss of Ig chain production. Analysis of the rescued IgMλ on Ultragel ACA 22 showed it to be predominantly in the pentameric form.

Monoclonal anti-idiotypes. For each patient, spent xenohybridoma culture supernatant containing rescued IgMλ was used as a source of immunogen for immunization of Balb/c mice (N.L.) or Lou rats (S.A.). Idiotypic IgM was prepared from supernatants as purified immune complexes with mouse or rat antihuman Fabμ serum by the method described for production of sheep polyclonal anti-idiotype.[12] Each animal was immunized with complexes containing 10 to 50 μg of idiotypic IgM in 0.2 mL CFA distributed among four subcutaneous sites. A booster intravenous (IV) injection of antigen in 0.2 mL aqueous solution was given 2 weeks later and immune spleen cells prepared three to four days after this. These were fused with NS-1 or 653 as for the rescue of IgM, and antibody-producing cloned hybridoma cells were expanded in spinner flasks.

Idiotypic IgM: Enzyme-linked immunosorbent assay (ELISA). This technique was used to monitor production of rescued human IgMλ from the xenohybrids and was carried out as described.[13] The coating antibody was rabbit antihuman μ-chain (10 μg/mL), and detection was with horseradish peroxide (HRP)–conjugated rabbit antihuman λ-chain (Dako, Copenhagen). A purified IgMλ macroglobulin was used as a standard.

For assessment of monoclonal anti-idiotypes a capture assay was used in which plates coated with rabbit antihuman μ-chain were treated with culture supernatant containing rescued IgMλ or normal IgM at 100 ng/mL. Undiluted anti-idiotype–containing culture supernatant was then loaded before detecting with HRP-rabbit antimouse Ig (1:1000) (Nordic Labs, Ltd, Maidenhead, Berkshire, UK) or HRP-sheep antirat Ig, as appropriate. Antibodies that showed strong reactivity with idiotypic IgM but no reactivity with normal IgM were selected for a second assessment by immunofluorescence on target cells. If binding to cells was inhibited by normal human serum, the antibodies were suspected to be against cross-reacting idiotypes, and further assessment by ELISA using panels of monoclonal IgMs and IgGs as antigens was carried out. An inhibition assay was established to quantitate the degree of cross-reactivity of the various anti-idiotypes with normal IgM or IgG. For this, idiotypic IgM was coated directly on a microtiter plate, and the mouse monoclonal anti-idiotype was mixed with varying amounts of homologous idiotypic IgM, cross-reacting idiotypic IgM, or normal IgM or IgG. The amount of mouse antibody able to bind to the coated antigen under these conditions was then measured by addition of HRP-rabbit antimouse Ig.

Preparation of IgM paraprotein from serum. The IgM paraproteins found to cross-react with anti-idiotypes were purified from patients' sera for use in the direct ELISA by chromatography on a column of Ultragel AcA 22 (LKB, Bromma, Sweden).[4] The two patients, Ha and Ru, each had a clinical picture of Waldenström's macroglobulinemia with an IgM serum paraprotein. Patient Ha had an IgMκ paraprotein at 53 g/L with cold agglutinin activity of anti-I type, and patient Ru had an IgMλ paraprotein at 16 g/L with no detectable autoantibody activity. In each case, since the paraprotein was present at high levels in the serum, separation on Ultragel AcA 22 was sufficient to yield purified pentameric IgM. Normal IgM used for screening hybridoma supernatants was prepared from pooled human serum by immunosorption as described previously.[4]

Immunofluorescence. Testing of the anti-idiotypes on the patient's dispersed tumor cells was carried out by the FACS III (Becton Dickinson Electronics, Mountain View, Calif). For inhibition studies, normal human serum (50 μL) was added to culture supernatants (50 μL) containing anti-idiotype at ~20 μg/mL before adding to the cells. Bound mouse or rat antibody was detected with fluorescent rabbit antimouse Ig or sheep antirat Ig as appropriate.

Tissue sections. Frozen tissue sections were used to assess reactivity of the anti-idiotypic antibodies with tonsil, reactive lymph node, and lymph nodes from patients with lymphoma. Cryostat sections (5 μm) were air dried at room temperature and stored at −70 °C until required. Immediately before staining, the sections were fixed in acetone for ten minutes and then stained by a two-layer immunoperoxidase technique[14] using HRP-rabbit antimouse Ig or HRP-sheep antirat Ig as the second layer and developing reaction product with diaminobenzidine tetrahydrochloride.

Cold agglutinins and hemagglutination inhibition. Other IgM paraproteins with or without cold agglutinin activity have been described previously[9] except proteins Cho, Dav, Ham, and Cab, which are noncold agglutinin proteins from patients with Waldenström's macroglobulinemia. For the indirect hemagglutination assay, the cold agglutinin Den, which is an IgMλ with anti-i activity, was coated to sheep RBC using tannic acid.[15] The anti-idiotypic antibody was then found to agglutinate coated cells, and using a convenient dilution of antibody (~0.3 μg/mL), the titers of proteins giving inhibition of agglutination were measured.

RESULTS

Preliminary screen for anti-idiotypes. Culture supernatants from growing clones were assessed by ELISA for anti-idiotypic activity, and those showing strong reactivity with the patient's idiotypic IgM and no reactivity against normal IgM were selected for expansion. Typically, out of five plates usually tested, supernatants from about 20% of the wells fulfilled this criterion of specificity.

The second part of the preliminary screen is to test for binding to target tumor cells by immunofluorescence in the presence or absence of normal human serum (NHS). The majority (≥90%) of supernatants reacting with idiotypic IgM in the ELISA also showed binding to target tumor cells; residual negative supernatants were not followed up, but apparent lack of binding could have been due to a low concentration. The results obtained for anti-idiotype against patient S.A. are shown in Figs 1 and 2. In Fig 1 the degree of fluorescence obtained with anti-Id (1) is seen to be unaffected by the presence of NHS or S.A. serum (2 and 3), indicating recognition of private determinants, whereas in Fig 2, the similar fluorescence obtained with anti–cross-reacting id (anti-IdX) (1) is completely abrogated by NHS or S.A. serum (2 and 3). The conclusion therefore is that the anti-IdX recognizes a minor component of normal Ig insufficient to be recognized in the ELISA, but sufficient in 50% NHS to inhibit binding to cells. The incidence of such antibodies generated is variable, with an average from seven successful fusions for anti-idiotype production of 10% to 15% of the selected anti-idiotypes. One anti-Id and one anti-IdX were then studied for each patient.

Secondary screen for anti-IdX. Using the capture assay and a random series of sera from patients with macroglobuli-

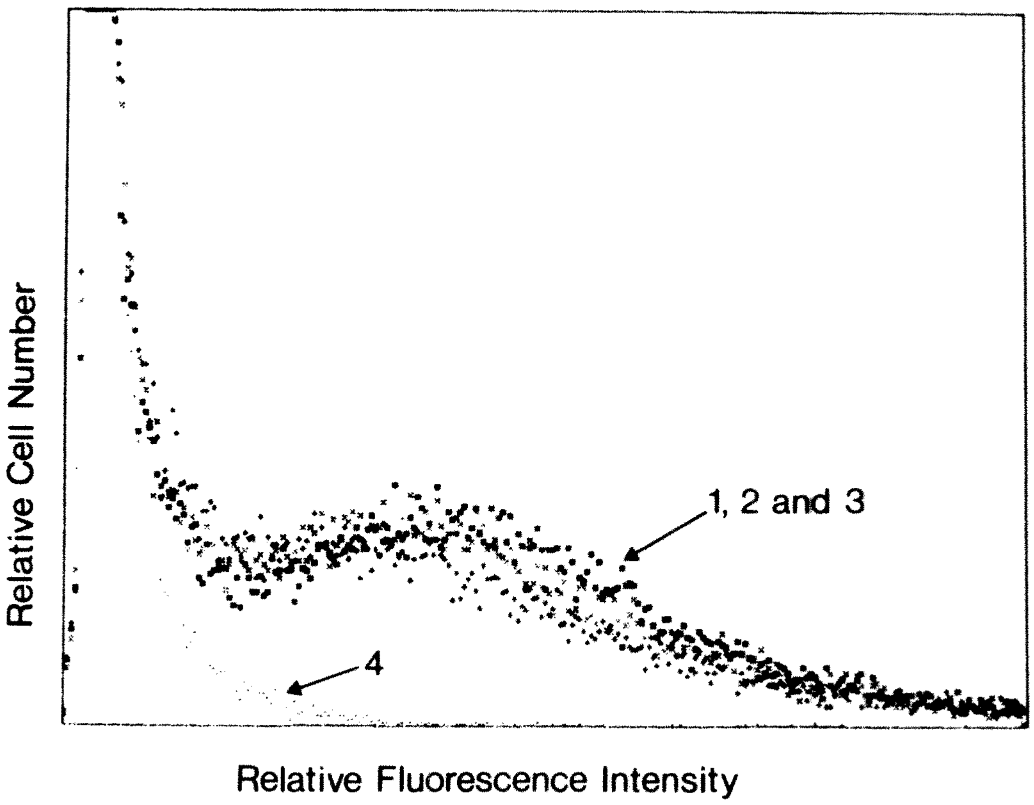

Fig 1. Binding of anti-idiotypic antibody raised against S.A. (anti-Id S.A.) to target lymphoma cells from S.A. and the effect of serum on this. Tumor cells were exposed to anti-idiotype (20 μg/mL) mixed with an equal volume of (1) phosphate buffered saline (PBS), (2) normal human serum, or (3) serum from S.A. Bound antibody was detected with fluorescent rabbit antirat IgG (0.5 mg/mL). (4) Control of normal rat IgG.

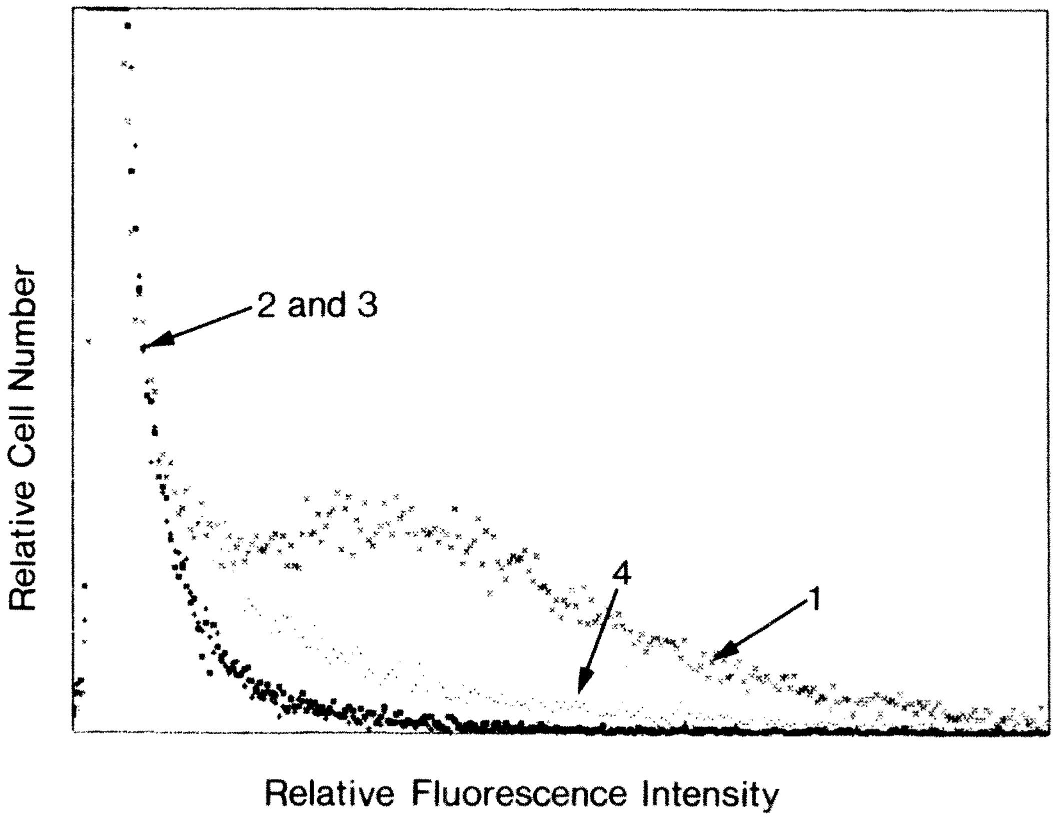

Fig 2. Binding of cross-reacting anti-idiotypic antibody raised against patient S.A. (anti-IdX S.A.) to target lymphoma cells from S.A. and the effect of various agents on this. Tumor cells were exposed to anti-idiotype (20 μg/mL) mixed with an equal volume of (1) PBS, (2) normal human serum, (3) serum from S.A., and (4) idiotypic IgMκ (1 mg/mL) from a patient with macroglobulinemia and cold agglutinin disease.

nemia or myeloma chosen for high levels of paraprotein and assayed at the same Ig concentration as homologous idiotypic Ig (100 ng/mL), the results shown in Table 1 were obtained. In this, the antibodies against anti-IdXs, defined by blocking of reactivity with homologous lymphoma cells in the presence of normal serum, show a limited degree of reactivity in the panel. The anti-IdX from patient S.A. recognized one of 13 IgM paraproteins, and this was an IgMκ with cold agglutinin activity. Since the idiotypic IgM from S.A. was IgMλ, this indicates no involvement of light-chain type in recognition. For the anti-IdX from patient N.L., three of 42 IgM paraproteins were positive, and these were one IgMκ and two IgMλs, again demonstrating that cross-reactivity is independent of light-chain type. The IgMκ recognized was different from that which reacted with the antibody against patient S.A. The anti-IdX from N.L. also reacted with an IgG paraprotein, demonstrating independence of the heavy-chain class also. Under the conditions of the assay, reactivities were strongly positive ($E_{492} > 1.0$) or negative, with no intermediate values.

Since anti-IdX S.A. recognized an anti-I cold agglutinin, a further investigation was made into reactivity with a panel of cold agglutinins directed against I or i antigens (Table 2). As shown, the antibody reacted with seven out of eight of the cold agglutinins, with the negative result being for IgM Ma, a protein known to lack the majority of idiotypic determinants shared by this family of cold agglutinins.[8,9] One of the proteins lacking cold agglutinin activity, but known to share idiotypic determinants with anti-I and anti-i cold agglutinins,[10] IgM Mar, also bound to the antibody. The idiotypic IgMλ rescued from the lymphoma cells of S.A. showed no cold agglutinin activity.

To localize the cross-reacting idiotypic determinants by a different procedure, the binding of anti-IdX to cells from patient S.A. was assessed in the presence or absence of the IgMκ cold agglutinin (Ha) or its Fabμ derivative prepared by papain digestion. Both the whole IgM and the Fabμ gave total inhibition of fluorescence when at 0.25 mg/mL, whereas they had no effect on binding by the non–cross-reacting anti-Id.

Quantitation of cross-reactivity. The inhibition ELISA used to measure the proportion of normal Ig recognized by anti-IdX S.A. is shown in Fig 3. For the assay, since the IgMκ macroglobulin recognized by the antibody reacted at the same level as the rescued IgMλ S.A., the former was coated onto the plate, and it can be seen that both this IgMκ and the rescued idiotypic IgMλ from S.A. are equally efficient at inhibiting the binding of anti-idiotype to the IgMκ on the plate, with ~4.5 ng/mL giving 50% inhibition (Table 2). However, normal IgM and IgG are considerably less efficient, and assuming equal efficacy of monomeric IgG and polymeric IgM in the assay, approximately one in 200 molecules of IgG and one in 600 molecules of IgM carry the cross-reacting idiotype. In a normal serum this would represent 60 μg/mL of IgG and 2 μg/mL of IgM. The fact that the curves for specific idiotype, cross-reacting idiotype, and

Table 1. Reactivities of Monoclonal Anti-idiotypes With Human Paraproteins

Anti-idiotype	Paraproteins IgM	 IgG
Anti-Id S.A.*	0/13	0/26
Anti-IdX S.A.†	1/13	0/26
Anti-Id N.L.*	0/42	0/26
Anti-IdX N.L.†	3/42	1/26

*Antibody against private idiotype.

†Antibody against cross-reacting idiotype.

The IgM paraprotein recognized by anti-IdX S.A. was not recognized by anti-IdX N.L.

Table 2. Hemagglutination Inhibition Assay Using IgM Den-Coated Red Cells and Anti-IdX From S.A.: Inhibition by Various Immunoglobulins

Protein	Minimum Concentration (μg/mL) Giving Complete Inhibition
Cold agglutinins	
Anti-I	
Low IgMκ	0.3
Step IgMκ	0.9
Sch IgMλ	0.3
Ha IgMκ	0.1
Da IgMκ	0.8
Ma IgMκ	> 50
Anti-i	
Den IgMλ	1.2
Galli IgMλ	0.3
Noncold agglutinins	
Mar IgMκ	0.7
Cho IgMλ	240
Ou IgMκ	262
Dav IgMλ	286
Ham IgMλ	> 48
Cab IgMκ	> 85

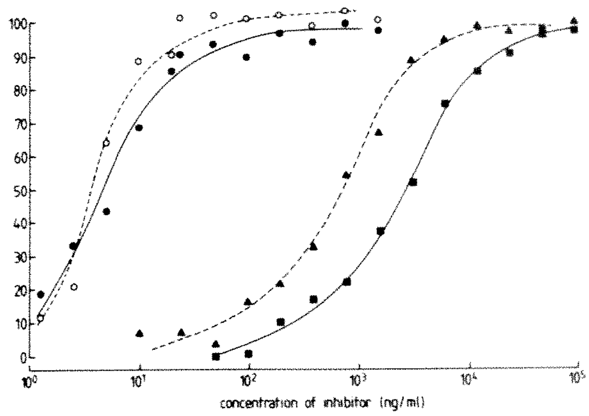

Fig 3. Inhibition ELISA to estimate the level of cross-reacting idiotype in normal IgM or IgG. The assay depends on cross-reacting idiotypic molecules in the normal Ig populations binding to the rat monoclonal anti-idiotype, thereby preventing its uptake by idiotypic Ig bound to the plate. Cross-reactive idiotypic IgMκ (200 ng/mL) was bound to microtiter plates and exposed to rat monoclonal anti-idiotype culture supernatant (1:300) to which varying amounts of inhibitor had been added. Bound rat antibody was detected with HRP-rabbit antirat IgG (1:500). Inhibitors used were --○--○-- IgM idiotype S.A.; -●-●-, cross-reacting IgMκ idiotype; --▲--▲-, normal human IgG; and -■-■-, normal human IgM.

normal IgG and IgM are parallel (Fig 3) strongly suggests that similar antigenic determinants are being recognized on all the competing Igs. There is no suggestion of interference by heterophile antibody in human serum that might recognize mouse Ig, and even if this were present, it should not interfere with the binding of anti-idiotype to idiotype.

Since the tumor cells from S.A. secrete no detectable Ig, it was possible to compare the abilities of normal serum from patient S.A. and pooled normal human serum to inhibit the aforementioned reaction. They were found to be equally effective, which suggests that the antibody is not anti-allotypic. This is also suggested by the heavy- and light-chain independence in the patterns of reactivity, leaving cross-reacting antigen in the variable region. Allotypic determinants in this region cannot easily be distinguished from framework determinants or less private idiotypic determinants.[16]

Comparable data, including the anti-allotype control, was obtained for the anti-IdX N.L., and the amounts required for the 50% inhibition are also shown in Table 3. The reason for the differences in concentrations of idiotypic IgMs required for 50% inhibition (4.5 and 460 ng/mL for S.A. and N.L. respectively) probably reflects the variable efficiency of the coating of the two IgM preparations to the plastic plate. This, however, does not affect the comparison of homologous and cross-reacting idiotypes in the competitive assays. In the case of N.L., the serum would represent a barrier of 23 μg/mL of IgG and 2 μg/mL of IgM.

Tissue reactivity. The anti-idiotypic antibodies were all tested on frozen sections of tonsil (three biopsies) and reactive lymph node (four biopsies). The two anti-Ids were negative on this tissue, whereas the two anti-IdXs stained a proportion of the mature cells in each case. The results for the two anti-idiotypes for S.A. are shown in Fig 4. In panel A, a monoclonal antibody specific for human μ-chains demonstrates B cells in the mantle zone of a reactive follicle; panel B shows the reactivity of anti-IdX S.A. with a proportion of these cells, and panel C shows no reactivity of anti-Id S.A. Similar results were obtained with anti-idiotypes to N.L.

Frozen sections of biopsy specimens from 12 patients with histologically defined monotypic B cell lymphoma were also tested with anti-idiotypes to S.A.; none reacted with anti-Id, whereas tumor cells from two patients reacted with anti-IdX. In both cases the tumor cells showed strong surface staining.

Table 3. Quantitation of Cross-reacting Idiotypic Ig by an Inhibition ELISA

Patient	Concentration Giving 50% Inhibition (ng/mL)				Cross-reacting Idiotype in Normal Ig (%)	
	IgM Id	IgM IdX*	nIgM†	nIgG	IgM	IgG
S.A.	4.5	4.5	2.8×10^3	7.5×10^2	0.2	0.6
N.L.	460	460	2.3×10^5	2.0×10^5	0.2	0.23

*Cross-reacting IgM idiotype.

†Normal IgM.

The ELISA depends on cross-reacting idiotypic molecules in the normal Ig populations binding to the monoclonal anti-idiotype, thereby preventing its uptake by idiotypic Ig bound to the plate. The concentrations giving 50% inhibition are calculated from the inhibition curves (see Fig 3).

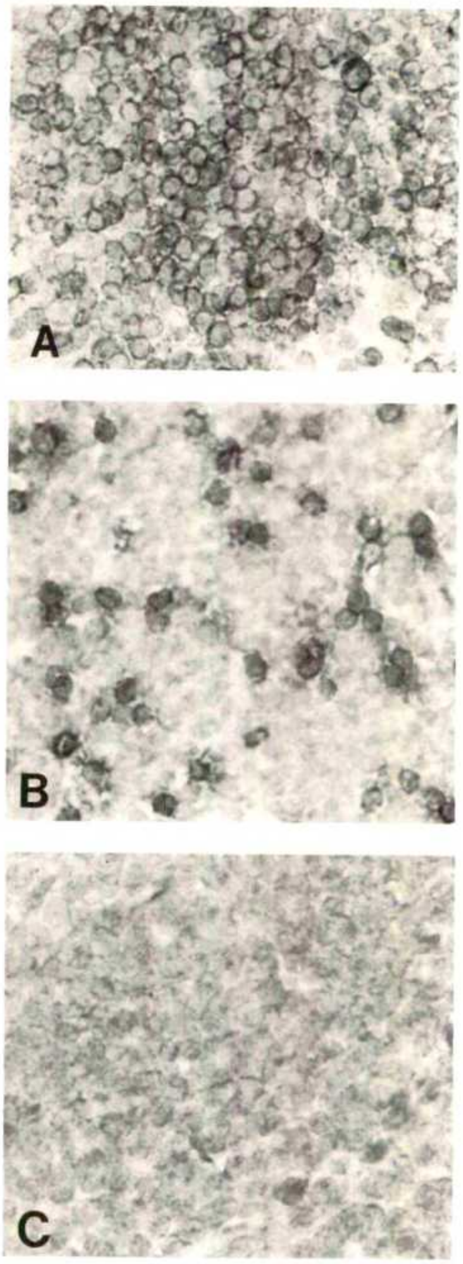

Fig 4. Reactivities of antibodies with normal lymphoid tissue. Frozen sections of reactive lymph node were treated with (A) mouse monoclonal antihuman μ-chain, (B) cross-reacting anti-idiotype S.A., and (C) specific anti-idiotype S.A. Binding was detected with HRP-rabbit antimouse or rat IgG as appropriate (Magnification × 500).

A similar analysis was carried out with anti-idiotypes to patient N.L. In this case neither anti-Id nor anti-IdX reacted with tumor cells in a further 12 biopsy specimens from patients with lymphoma, although a few of the residual normal cells reacted as expected. The anti-idiotypes also did not react with the biopsy specimen from patient S.A.

DISCUSSION

Since the description of attempted therapy of human B cell lymphoma with anti-idiotypic antibody[1] and particularly since the reported apparent success in one patient,[2] a number of groups have raised such antibodies for treatment. However, as well as problems of mechanisms of tumor cell escape from antibody attack,[3] it has been shown that the presence of idiotypic IgM in the plasma, arising from low levels of secretion by the neoplastic cells,[4] is a disadvantage in the application of this therapy. Anti-idiotype must traverse the serum to reach the tumor cells, and if it is consumed by idiotype, more antibody is required, and the immune complexes generated can consume effector mechanisms and perhaps cause toxic reactions.[5]

Theoretically the situation is worse if the antibody recognizes more public idiotypes since they will correspondingly be present in the normal immunoglobulin, and it can be envisaged that antibodies to increasingly public idiotypes will recognize more lymphomas but also more of the serum Ig. In view of this, the screening method in this laboratory examines reactivity of antibody with target cells in the presence of normal serum. Clearly the screening procedure must be set in an appropriate way to select the antibody specificities desired, and this is the reason for the two-stage assessment using first, either idiotypic IgM or normal IgM as assessed by ELISA, followed second by the test on target cells in the presence of serum. The cross-reacting anti-Ids pass the first test but fail the second. It is difficult to assess why other groups produce different proportions of such specificities, and sometimes no cross-reacting anti-Ids[17] but differences in the nature of the immunogen and in immunization and screening methods must be considered. Immunization with soluble idiotypic IgM rather than cells should give a larger range of antibodies that can be surveyed for such activity; this is probably necessary since the incidence in this study was 10% to 15% of putative anti-idiotypes. Some investigators have used screening against a variety of lymphomas and normal cells by immunofluorescence, which is not rigorous, and antibodies to cross-reacting idiotypes have been selected and proposed for therapy with no measure of the serum barrier that exists.[18]

However, the attraction of using a single anti-idiotype for more than one patient remains and has led us to establish a test for the degree of cross-reactivity shown by the numerous antibodies (10% to 15% of selected anti-idiotypes) that fail our test in the presence of serum. Two typical examples have been analyzed, and the extra barrier that must be overcome for these antibodies to traverse the serum has been quantitated. If such measurements are made, it might be possible to create a panel of the more specific end of the spectrum, eg, those antibodies that recognize an arbitrary value of <30 $\mu g/mL$ of normal IgG, with a view to therapy and, certainly, to use them for monitoring tumor cell spread. Such a barrier should not create major problems for antibody infusion, and it could be lowered further by plasmaphoresis.[1,5] For application of the test in which rescued idiotypic Ig has not been prepared, Fab'γ prepared from the surface Ig of intact cells or a tumor cell lysate[12] could be used. Since such antibodies probably recognize framework determinants in the variable region rather than those involved in putative antigen-binding (CDRs), it might be of interest to see whether the expression of such determinants is affected by mutational events at the same frequency as appears to occur for more private idiotypes.[19]

A further bonus of investigating these antibodies is the finding of unexpected activities such as that shown by the anti–cross-reacting idiotype produced against idiotypic IgM from patient S.A. that appears to recognize cold agglutinins. The pattern of reactivity seen is very similar to that of a monoclonal antibody[20] specifically raised against IgM Mar known to express such cross-reacting idiotypic determinants.[10] Such reagents might provide useful insights into the range of idiotypic determinants found in lymphoma and autoimmune diseases in relation to those in the normal immunoglobulin pool.

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Production and Characterization of a Monoclonal Antibody Reactive With a Specific Neoantigenic Determinant (Comprising Bβ 54-118) in Degradation Products of Fibrin and of Fibrinogen

P.W. Koppert, J. Koopman, F. Haverkate, and W. Nieuwenhuizen

Balb/c mice were immunized with a mixture of fibrin degradation products (XDPs) prepared by complete lysis of a human blood clot by tissue-type plasminogen activator and purified by immunoaffinity chromatography. Spleen cells of the mice were fused with P3 × 63 Ag 8653 myeloma cells. A clone (FDP 14) was selected that produces monoclonal antibodies (MoAbs) of the IgG1κ type that react with a neoantigenic determinant exposed in these XDPs, but not in intact fibrinogen or in fibrin monomers. Furthermore, the MoAb is reactive with some pure, individual degradation products of fibrinogen (fragments X, Y, E, and the N-terminal disulphide knot) and with the fibrinogen Bβ-chain but not with Aα- and γ-chains or with fragments D, FCB-2 and FCB-3. Comparison of the known primary structures of these fibrinogen fragments indicates that the stretch Bβ 54-118 comprises at least an important part of the epitope recognized by FDP-14. Apparently, this stretch contributes importantly to a neoantigenic determinant that is not functional in intact fibrinogen and fibrin monomer and that can be made functional by reduction of fibrinogen, or by digestion with plasmin or CNBr.

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FIBRIN is the insoluble protein matrix of a blood clot. Fibrin has a temporary function; after it has fulfilled its role in hemostasis and tissue repair, it is proteolyzed by plasmin. This process, designated as fibrinolysis, yields a mixture of soluble fibrin degradation products (XDPs). In principle, fibrinolysis could be assessed from the concentration of XDPs in plasma. However, even in conditions of strongly activated fibrinolysis, levels of XDPs will be in the submicromolar range, ie, they are best assessed by sensitive, immunologic methods.

Most existing assays are based on polyclonal antibodies and are carried out in serum to avoid the cross-reactions of polyclonal antibodies with fibrinogen in plasma. The use of serum, however, often yields falsely positive (eg, by incomplete clotting or by fibrinolysis during serum preparation) or falsely negative (eg, since some XDPs tend to adsorb to fibrin) results. Thus, assays should preferably be carried out in plasma or blood.

With monoclonal antibodies (MoAbs), the problems inherent with polyclonal antibodies can be overcome, especially since neoantigenic (degradation product–specific) determinants have been shown to occur in fibrin(ogen) degradation products.[1-7]

This paper describes the production and characterization of a MoAb specific for the degradation products of both fibrin and fibrinogen and not reactive with intact fibrinogen and fibrin monomers.

MATERIALS AND METHODS

Trasylol was bought from Bayer Pharmaceuticals (Mijdrecht, The Netherlands), horseradish peroxydase (HRP) from Boehringer Mannheim Biochemicals (Mannheim, FRG), and thrombin from Leo (Copenhagen). Diisopropylfluorophosphate (DFP) was purchased from Sigma Chemical Co (St Louis) and was dissolved in dry isopropanol to a concentration of 0.1 mol/L. Tissue-type plasminogen activator (t-PA) was prepared by Drs D.C. Rijken and J.H. Verheijen as described before.[8]

Preparation of a lysate of clotted whole blood. A whole blood clot lysate was prepared according to Koopman and Haverkate.[9] Whole blood from a healthy volunteer was collected in a plastic tube and mixed quickly with a solution of t-PA (final concentration, 90 IU/mL). The mixture was transferred to a glass tube containing thrombin (final concentration, 1 NIH/mL) and incubated at 37 °C. After complete lysis (two hours) of the formed clot, Trasylol (final concentration, 100 KIU/mL) and DFP (final concentration, 1 mmol/L) were added. After removal of cells by centrifugation at 2,300 g for 20 minutes at room temperature, the supernatant, designated as blood lysate, was collected and stored at −20 °C.

Preparation of the immunoaffinity column. Immunoglobulins (IgGs) were purified by sodium sulphate precipitation[10] from a pool of different polyclonal rabbit antisera against fibrinogen, fibrin monomers, fragments X, Y, D, E, and D-dimer.[11] The isolated IgGs were coupled with activated CH Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturer's instructions. The amount of IgG coupled per milliliter of wet gel was 5 mg. This was calculated from the difference in IgG concentration before and after the coupling reaction ($A^{1\%}_{1\,cm}$ = 14.5 at 280 nm), after dilution of the gel supernatant with 1 mol/L HCl. The immunoadsorbent was washed and stored at 4 °C in borate buffer (6 mmol/L disodium tetraborate, 0.2 mol/L boric acid, 5 mmol/L Na_2 EDTA), pH 7.4, containing 20 KIU of Trasylol per mL and 0.01% (wt/vol) of sodium azide.

Purification of XDPs from the blood lysate. One milliliter of the blood lysate was diluted with 3 mL of borate buffer, applied to a 2-mL column of immunosorbent, and washed sequentially with 100 mL of borate buffer, 20 mL of borate buffer containing 0.5 mol/L NaCl, and 20 mL of tenfold-diluted borate buffer. The XDPs were eluted with 0.01 mol/L NaOH, immediately neutralized with 0.1 mol/L HCl, then dialyzed against distilled water and lyophilized.

Other antigens. Human fibrinogen was purified as described previously.[12] The Aα-, Bβ- and γ-chains were separated by reduction and carboxymethylation of fibrinogen and purified as described by Doolittle et al.[13] Plasmin-generated fibrinogen degradation products (FDPs) were prepared and purified as described by Nieuwenhuizen et al for fragment X[14] and fragment Y[15] and by Van Ruijven-Vermeer et al for fragments D_{cate}, D_{EGTA}, and E.[11] The aminoterminal disulphide knots (NDSK) of fibrinogen and fibrin were prepared and purified as described by Blombäck et al[16] and Olexa and Budzynski,[17] respectively.

Fragment FCB-2[18,19] (also known as Hol-DSK[20]) and fragment FCB-3[19] were purified as described before.[18] The constituent chain remnants of these fragments are summarized in Table 1. Fibrin monomers were prepared as described by Haverkate and Timan.[21]

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Table 1. Summary of the Chain (Remnant) Composition of Fibrin(ogen)-Derived Fragments

	Amino Acids Stretches Derived From		
Product	Aα-Chain	Bβ-Chain	γ-Chain
Fibrinogen	$(1–610)_2$	$(1–461)_2$	$(1–410)_2$
Fibrin monomer	$(17–610)_2$	$(15–461)_2$	$(1–410)_2$
X	$(1–208)_2$	$(1–461)_2$	$(1–410)_2$
Y	1–208/1–78	1–461/54–122	1–410/1–58
D_{cate}	111–197	134–461	86–410
E_3	$(1–78)_2$	$(54–122)_2$	$(1–58)_2$
NDSK (Fibrinogen)	$(1–51)_2$	$(1–118)_2$	$(1–78)_2$
NDSK (Fibrin)	$(17–51)_2$	$(15–118)_2$	$(1–78)_2$
FCB-2	148–207	191–224 225–242 243–305	95–265
FCB-3	241–476	—	—

The figures indicate the numbers of amino acids.

HRP conjugate of IgGs against fibrinogen-related material. The same pool of polyclonal antisera against fibrin(ogen) and degradation products previously described to prepare the XDP immunoadsorbent was used. For conjugation, however, IgG were purified using caprylic acid (Aldrich Chemical Co, Beerse, Belgium) instead of sodium sulphate. To 10.0 mL of the pooled antisera, 20.0 mL of a solution containing 0.06 mol/L sodium acetate, pH 4.0, was added. Then 0.68 mL caprylic acid was added dropwise under continuous stirring at room temperature, and the mixture was stirred for 30 minutes. The precipitate formed was removed by centrifugation for 30 minutes at 25,000 g at room temperature. The IgG-containing supernatant was filtered through glass-wool, dialyzed against 0.01 mol/L NH_4HCO_3 and lyophilized. The IgG's were conjugated with HRP using *N*-succiminidyl-3-(-2 pyridylthio) propionate (SPDP, Pharmacia) as a coupling agent according to Pharmacia's instructions. After conjugation excess HRP was removed by gel filtration on Sephacryl S-200 in 0.3 mol/L sodium chloride, pH 7.5.

Immunization scheme. Female Balb/c mice (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands) were injected intraperitoneally with 25 μg purified XDPs in Freund's complete adjuvant (Difco Laboratory, Detroit) and then at 3-week intervals with 25 μg XDPs in incomplete Freund's adjuvant. An intravenous injection of 25 μg XDPs in phosphate buffered-saline (PBS) was given three days before fusion.

Fusion. Fusion was performed as described before.[22] Spleen cells (8×10^7) of immunized mice were fused with 2×10^7 myeloma cells, P3 × 63 Ag 8653, in 40% polyethylene glycol (PEG) 4000 (Baker Chemical Co, Deventer, The Netherlands). Growth and selection media were as described by Köhler and Milstein.[23] The fusion mixture was divided over 240 wells of 96-well microtiter plates. Wells with growing cells were screened for antibody production by sandwich enzyme immunoassay (EIA) as will be described. Antibodies producing hybridomas were cloned and recloned by limiting dilution (0.5 cells/well).

Hybridoma selection procedures. Hybridoma's were selected using two different procedures. (1) The enzyme-linked immunosorbent assay was performed essentially as described before[22] with fibrinogen or fragment Y as immobilized antigens. The latter was chosen as a model for degradation products. Immunoreactive mouse immunoglobulin was visualized with HRP-labeled rabbit antimouse immunoglobulin from Nordic Immunology (Tilburg, The Netherlands) using 3, 3', 5, 5'-tetramethyl benzidine (TMB) from Aldrich Chemical Co, Milwaukee, and H_2O_2 as the substrate mixture.[24] Criteria for selection were a negative reaction with fibrinogen and a positive reaction with fragment Y. (2) A double-sandwich EIA was developed because it was our experience that some antigens lose or expose determinants when adsorbed directly to the plastic wall of a microtiter plate. For this reason supernatants of hybridoma cultures were also screened for a positive reaction with XDPs and pure, individual fibrinogen degradation products (FDPs) such as X and Y (FDPs) and a negative reaction with intact fibrinogen in a double-sandwich EIA. For this assay quantities of 120 μL of a 10 μg/mL solution of rabbit antimouse Ig (7S) (Nordic) in 0.01 mol/L PBS, pH 7.4, were pipetted into the wells of a microtiter plate (Immulon, Dynatech Laboratories, Inc, Plochingen, FRG). The plates were incubated for 16 hours at 4 °C. Before use, the plates were washed three times with PBS containing 0.05% Tween 20 (PBS/Tween). Then 100-μL aliquots of cell culture media were pipetted into the wells and incubated for 30 minutes at room temperature. After three washings with PBS/Tween, 100-μL portions of fibrinogen or fragment Y solution (5 μg/mL PBS containing 0.1% Tween 20) were added to each of the wells and incubated for 30 minutes at room temperature. After washing, the wells were filled with a solution of the polyclonal rabbit anti-FDP antiserum conjugated with HRP (see the previous material) and incubated for 30 minutes at room temperature. Detection of immobilized conjugate was done using the TMB substrate solution as described in the ELISA procedure.

Subclass assessment and purification of the MoAb. Subclass assessment was carried out by immunoelectrophoresis (IEF) as described by Radl.[25] Antisera against mouse α-, μ-, δ-, ε-, γ-, κ-, and λ-chains were gifts from Dr J. Radl (Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands).

Purification was performed on CL Sepharose 4B/protein A (Pharmacia) according to Ey et al.[26] Ascites fluid was diluted five times in 0.01 mol/L PBS, pH 8.0, and applied to the column. Nonbound material was eluted by this buffer. The MoAb was eluted with 0.1 mol/L sodium citrate/citric acid (pH 5.0). Occasionally, the MoAb was purified by the Affigel/protein A monoclonal antibody purifying system (MAPS) procedure of Bio-Rad Laboratories (Richmond, Calif) according to the manufacturer's instructions. Immunoreactivity was checked by ELISA, purity by IEF. In some cases the MoAb solution was concentrated by dialysis against solid PEG 20,000. MoAb solutions were stored at −20 °C.

Determination of the epitope. The epitope recognized by the MoAb on XDPs and FDPs was identified using different pure fibrin(ogen)-derived fragments, by ELISA, the double-sandwich EIA, and by the cross-reactivity assay (CRA) according to Soria et al.[27] In this latter assay, a dilution of MoAb solution is chosen that gives 80% of the maximum response in the assay as described in the ELISA procedure on fragment Y–coated plates. Antibody is preincubated (16 hours, 4 °C) at this concentration with different concentrations (0 to 200 μg/mL) of different antigens. After preincubation, an aliquot of the incubation mixture is transferred to the wells of a fragment Y–coated plate for ELISA. A response decreasing with increasing antigen concentrations indicates a reaction with the antigen used in the preincubation step.

RESULTS AND DISCUSSION

Three weeks after fusion and selection, cell growth was observed in 197 of the 240 seeded wells. The media of only three wells reacted with fragment Y and not with fibrinogen. After cloning and recloning, 30 stable clones were obtained. One is described in this paper. This line has been in culture for 1 year and has been injected into the peritoneal cavity of Balb/c mice for in vivo production in ascites fluid. Also, in vivo the clone is very stable, ie, more than 6 months (seven passages). We will refer to the MoAb produced by this clone as FDP-14. Subclass assessment showed that FDP-14 is of the IgG1κ type. This made it possible to isolate the immunoglobulins by CL Sepharose 4B/protein A chromatography. Table 2 summarizes the immunoreactivity of FDP-14 in ELISA with fibrinogen, with 13 fibrinogen-derived fragments, and with the XDP mixture used to immunize the Balb/c mice. Some antigens tend to react with a MoAb only when adsorbed to the plastic of a microtiter plate and not in solution (J. and C. Soria, P.J. Gaffney, personal communication; personal observation). To exclude this possibility we also performed double-sandwich EIAs and CRAs in addition to the ELISA. We observed that FDP-14 is reactive with the same antigens in ELISA, in double-sandwich EIA, and in CRA (Table 2), indicating that the FDP-14 epitope is functional and accessible in both adsorbed antigens and antigens in solution.

It could be argued that the XDPs that were used as immunogen might have contained fragments of, eg, fibronectin or α_2-antiplasmin cross-linked to the chains of the XDPs. These fragments could contribute to a neoantigenic determinant. This is highly unlikely, however, because fragments X and Y from purified fibrinogen are also reactive but are not cross-linked and have been purified by methods completely different from those used to isolate XDPs. Moreover, fragment E does not contain cross-linking sites. The specificity of FDP-14 for fibrin(ogen) degradation products is further demonstrated by the CRA results shown in Fig 1. This Figure shows that complete inhibition is obtained with any of the studied pure fragments that are reactive in ELISA and in double-sandwich EIA.

The smaller potency of Bβ-chain and NDSK (Table 2) in CRA is probably only apparent and due to the rather low solubility of these fibrinogen derivatives. The concentrations of Bβ and NDSK giving 50% inhibition in CRA are calculated concentrations, and the actual concentrations of dissolved Bβ and NDSK are not known. Fragments X, Y, and E from different batches were always positive in ELISA and double-sandwich EIA. However, the concentrations required for 50% inhibition in CRA varied from batch to batch between 10^{-8} and 10^{-9} mol/L. For that reason we could not calculate dissociation constants (KDs) for these soluble fragments, but we guess from our data that they are on the order of 10^{-8} to 10^{-9} mol/L.

Table 2. Immunoreactivity of MoAb FDP-14 With Fibrinogen and Derivatives in ELISA, Double-Sandwich EIA, and in CRA (Typical Experiment)

Antigen	Response in ELISA and Double-Sandwich EIA	50% Inhibition in CRA With
Fibrinogen	–	—*
Fibrin monomers	–	—*
Fragment X	+	8×10^{-9} Mol/L
Fragment Y	+	3×10^{-9} Mol/L
Fragment D_{cate}	–	—*
Fragment E_3	+	2×10^{-9} mol/L
XDP mixture	+	1×10^{-9} mol/L†
Aα-chain	–	—*
Bβ-chain	+	10^{-6} mol/L‡
γ-chain	–	—*
FCB-2	–	—*
FCB-3	–	—*
NDSK (fibrinogen)	+	10^{-6} mol/L‡
NDSK (fibrin)	+	10^{-6} mol/L‡

*More than 10^{-3} mol/L needed for 50% inhibition.

†See legend to Fig 1.

‡Calculated from the amounts actually added, not corrected for the nondissolved fraction.

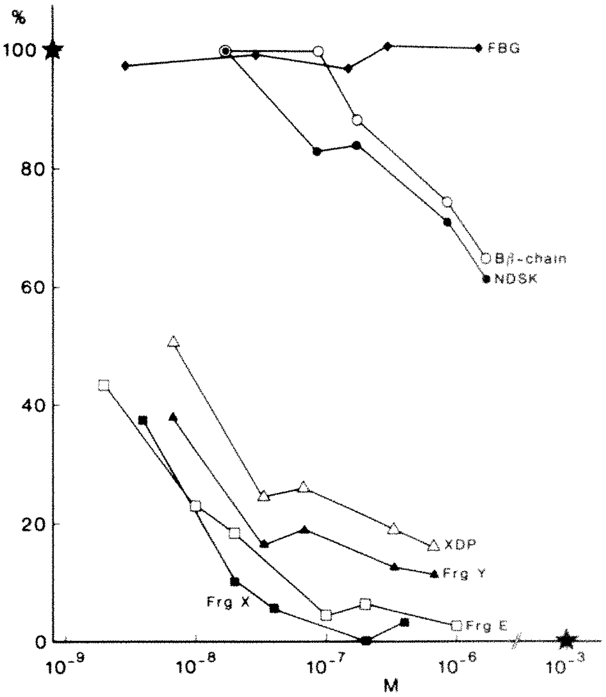

Fig 1. **Results of a typical CRA (details in text) of fibrinogen and pure derivatives. Ordinate, response remaining in CRA (100% response corresponds with 80% of the maximum response in ELISA). The concentration of XDPs has been expressed in moles per liter, assuming that XDP is predominantly a mixture of repeating Y (molecular weight, 170,000) subunits held together by cross-links. *Designates the points at which all lines come together.**

Figure 2 shows the time-dependent changes of dose-response curves of normal human plasma upon treatment with streptokinase. In the EIA used in Fig 2 (which will be published in full detail elsewhere), FDP-14 is used as the catching antibody and a HRP conjugate of a MoAb specific for fibrinopeptide A–containing fibrinogen fragments (Y18[22]) as the tag. Figure 2 demonstrates that a pool of normal plasmas is not reactive with FDP-14, but upon SK treatment, the response in the EIA increases with time. At all times the dose-response curves are parallel.

The results in Table 2 show that FDP-14 is not reactive with intact fibrinogen and fibrin monomers. The positive reaction with the Bβ-chain and the lack of reaction with Aα- or γ-chain suggest that the epitope for FDP-14 is composed primarily from residues of the Bβ-chain. On the basis of our results, however, contributions from residues of Aα- and/or γ-chains cannot be excluded. Thus, residues on the Bβ-chains may be necessary but not sufficient for a complete epitope. Another possibility is that the epitope is not in the

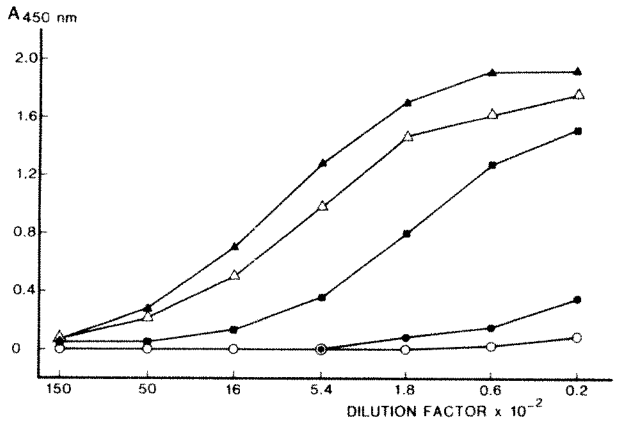

Fig 2. Time-dependent changes of dose-response curves of normal plasma upon streptokinase (SK) treatment in an EIA in which FDP-14 was used as the catching antibody; Y18[22] conjugated with HRP was the tag. To 0.6 mL of normal pooled plasma (37 °C) was added 0.01 mL of an 0.05 mol/L EDTA solution and SK (Kabikinase, KABI, Uppsala, Sweden) to give a final concentration of 30 Ploug units per milliliter. Samples were taken at timed intervals and reactions in these samples stopped with aprotinin (final concentration, 160 KIU/mL). These samples were used to make dilution curves. Key: ○, t = 0; ●, t = five minutes; ■, t = 15 minutes, △, t = 45 minutes; and ▲, t = 60 minutes.

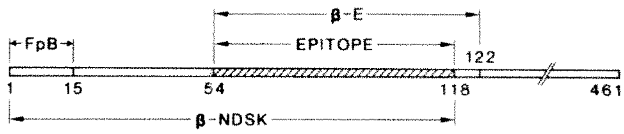

Fig 3. Position of the stretch essentially contributing to the epitope of MoAb FDP-14 on the Bβ-chain (residues 1 to 461). Bβ-chain remnants of NDSK (Bβ 1 to 118) and fragment E (Bβ 54 to 122) are designated as β-NDSK and β-E, respectively.

optimal conformation in isolated Bβ-chains and in NDSK. In fact, important constituents of the FDP-14 epitope may be accessible even in intact fibrinogen, but in a conformation that is not functional, ie, not recognized by FDP-14. Since fragments E and NDSK react with FDP-14 and since the fibrinogen Bβ-chain remnants in these fragments comprise residues Bβ 54 to 122 and Bβ 1 to 118, respectively (Table 1), these results strongly suggest that amino acids on the Bβ-chain between residues 54 and 118 are essential constituents of the epitope reactive with FDP-14 (Fig 3) and that this Bβ-chain stretch at least contributes importantly to a neoantigenic determinant in fibrin(ogen)-derived fragments.

Several antigenic determinants have been described in the Bβ-chain stretch 1 to 118.[28] Cierniewski and Edgington, using polyclonal antibodies directed against Bβ 1 to 118,[29] have presented indirect evidence that neoantigenic determinants may be present in degradation products on the stretch Bβ 54 to 118, ie, these determinants are exposed on isolated Bβ 1 to 118, but buried in intact fibrinogen. Recently, Pandya et al[30] have shown that Bβ 1 to 42 is cleaved selectively from fibrinogen upon treatment with protease III from *Crotalus atrox* venom and that this cleavage is not accompanied by a measurable conformational change in fibrinogen. They concluded that Bβ 1 to 42 in fibrinogen is surface exposed and accessible to IgG molecules and to proteases. Our results may also be explained, assuming that at least the part of the stretch Bβ 54 to 118 that contributes significantly to the FDP-14 epitope is not accessible to IgG molecules in intact fibrinogen and fibrin monomers and that cleavage of the Bβ 53 to 54 bond (and major parts of the carboxy-terminus of the Aα-chains) exposes the FDP-14 epitope on Bβ 54 to 118. This would corroborate the conclusions drawn by Cierniewski and Edgington.[29] Our results show that this determinant can be brought to full expression, by plasmin degradation and at least partly by CNBr cleavage of fibrin(ogen) or by reduction of fibrin(ogen).

FDP-14 is different from other MoAbs, which also have their epitopes on the Bβ-chain. Hui and co-workers[31] raised monoclonals against the first seven amino acids of the fibrin β-chain, which, as we have shown here, have no relation with the FDP-14 epitope. Kudryk et al[32,33] described two monoclonals. One (MoAb/1-8C6[32]) is of the IgG2a type, whereas FDP-14 is an IgG1. Furthermore, MoAb/1-8C6 crossreacts with fibrinogen and has its epitope around the thrombin cleavage site in the Bβ-chain, ie, distant from the FDP-14 epitope. The other one, MoAb/T2Gls,[33] reacts with Bβ 15 to 42 and weakly with fibrinogen, NDSK of fibrinogen, and with NDSK from which only the fibrinopeptides A have been removed. MoAb/T2Gls shows, however, a strong reaction with NDSK after thrombin treatment, ie, when both fibrinopeptides A and B have been cleaved off. In contrast, FDP-14 reacts strongly with fragments E_3 in which Bβ 15 to 42 is absent (Table 1), does not react with fibrinogen, and does not discriminate between NDSK with or without fibrinopeptides. These data show that FDP-14 is a completely new MoAb with no resemblance to previously described MoAbs.

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Separation and Analysis of Subcellular Organelles in a Human Promyelocytic Leukemia Cell Line, HL-60: Application to the Study of Myeloid Lysosomal Enzyme Synthesis and Processing

By William M. Nauseef and Robert A. Clark

We describe a system for analysis of the intracellular pathways in the biosynthesis and packaging of functionally important proteins in human myeloid cells. The human promyelocytic cell line HL-60 was used since peripheral blood neutrophils are terminally differentiated and do not actively synthesize protein. Cells were disrupted by nitrogen cavitation and subcellular organelles in postnuclear supernatant separated on a discontinuous gradient of Percoll modified to resolve organelles important in protein synthesis. This Percoll gradient separated azurophilic granules from less dense organelles and partially separated the less dense organelles from one another. Approximate densities of organelles identified by electron microscopy and by biochemical markers are azurophilic granules, 1.102 g/mL; endoplasmic reticulum, 1.039 g/mL; Golgi apparatus, 1.032 g/mL; and plasma membrane, 1.027 g/mL. We validated the utility of this method of subcellular fractionation by examining intracellular transport of myeloperoxidase, a myeloid lysosomal enzyme present in azurophilic granules. The subunits of mature myeloperoxidase (molecular weight [mol wt] = 59,000 and 13,500) cosediment with biochemical markers for lysosomes, whereas the large-mol wt (89,000) precursor forms cosediments with biochemical markers of less dense organelles. Within the limits of assay sensitivity, the 89,000-mol wt precursor is enzymatically inactive and has no spectral evidence for a heme group, suggesting that precursors of myeloperoxidase may undergo proteolytic maturation in a prelysosomal compartment with concomitant incorporation of a heme group and acquisition of enzymatic activity. This system of analysis should be suitable for the identification, subcellular localization, and maturational analysis of other myeloid lysosomal enzymes as well as functionally important membrane proteins.

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THE MICROBICIDAL and tumoricidal capacity of human polymorphonuclear neutrophils (PMNs) depends on the interaction of toxic oxygen radicals such as hydrogen peroxide, superoxide anion, and hydroxyl radical with granule proteins, most notably myeloperoxidase (MPO), to generate a toxic milieu within the phagolysosome.[1] Many aspects of this complex system have been characterized in detail, although studies of the biosynthesis and intracellular transport of myeloid lysosomal enzymes have been few in number.[2-6] The major limitation for such studies has been the paucity of protein synthesis in cells as terminally differentiated as are human PMNs.[7] For this reason, investigators have recently used cell lines derived from human tumors for study of various processes in myeloid cells.[8] One such line, the human promyelocytic leukemia line HL-60,[9,10] is especially suited for such studies because these cells resemble promyelocytes and can be chemically induced to develop the morphologic and functional characteristics of mature myeloid cells such as PMNs[11] or macrophages.[12] HL-60 cells contain azurophilic granule proteins such as MPO,[13] elastase,[13] β-glucuronidase,[11] and cathepsin B[14] and after induction display functional membrane receptors,[15,16] respond to a chemotactic stimulus,[17] carry out phagocytosis,[18] and exhibit a stimulus-dependent respiratory burst.[19]

To dissect the subcellular pathway for transport of nascent lysosomal enzymes and functionally important integral membrane proteins, we have developed a method of subcellular fractionation using nitrogen cavitation and density centrifugation on a Percoll gradient. This method allowed clear separation of lysosomes and less dense organelles important in protein synthesis and posttranslational modification of nascent proteins. We illustrate the utility of this method of analysis by examining MPO synthesis, demonstrating that the enzymatically inactive, large-mol wt precursor form of MPO cosediments with Golgi apparatus, whereas enzymatically active, heme-containing mature MPO cosediment with biochemical markers for lysosomes.

MATERIALS AND METHODS

HL-60 cells. The HL-60 cell line was a gift from Dr H.L. Malech of Yale University and was originally obtained from Dr R.C. Gallo at the National Institutes of Health. Cells were cultured in RPMI 1640 (provided by the Cancer Center, University of Iowa) supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L glutamine, and penicillin-streptomycin. Cells were determined to be free of mycoplasma infection.

Disruption of cells. Between 1 and 2 × 10^9 HL-60 cells were harvested, washed once in phosphate-buffered saline (150 mmol/L NaCl, 10 mmol/L potassium phosphate, pH 7.0, PBS), and suspended in 20 mL of ice-cold relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 1 mmol/L adenosine triphosphate-Na_2, 3.5 mmol/L $MgCl_2$, 10 mmol/L PIPES, pH 7.3). The cells were pressurized with N_2 for 20 minutes at 350 psi in a nitrogen bomb (Parr Instrument Co, Moline, Ill) at 4 °C and the cavitate collected dropwise into ethylene glycol tetra-acetic acid (EGTA), pH 7.4 (final concentration, 1.25 mmol/L), as previously described.[20]

Subcellular fractionation. The cavitate was centrifuged (500 g for ten minutes at 4 °C) to separate nuclei and unbroken cells (P_1) from the supernatant (S_1). S_1 was layered atop the preformed Percoll gradients.

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Density centrifugation. Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) was made isotonic by adding one tenth the final volume of a tenfold-concentrated solution of relaxation buffer (10× relaxation buffer) containing 12.5 mmol/L EGTA. Two Percoll solutions, a light and a dense, were prepared. To prepare 100 mL of light Percoll (final density, 1.040 g/mL), 10 mL of 10× relaxation buffer with EGTA, 26.4 mL of undiluted Percoll (density, 1.130 g/mL), and 63.6 mL of deionized water were mixed. To prepare 30 mL of dense Percoll (final density, 1.120 g/mL), 3.0 mL of 10^{x} relaxation buffer, 26.4 mL of undiluted Percoll, and 0.6 mL of deionized water were mixed. To form the gradient for separation of subcellular organelles, 22 mL of light Percoll were layered atop 6 mL of dense Percoll and the tube centrifuged at 48,000 g (JA-20 rotor, Beckman Instruments, Inc, Palo Alto, Calif) for 15 minutes at 4 °C. Subsequently, S_1 was layered atop the preformed gradient and centrifugation continued for an additional 15 minutes at 48,000 g. Two additional gradients were run in parallel: one (blank gradient) loaded with a volume of relaxation buffer equal to that of S_1 loaded on the sample gradient, and one (marker gradient) loaded with colored marker beads of known density (Pharmacia) suspended in an equal volume of relaxation buffer. Distances from the bottom of the tube to bands seen in the sample gradient and to density beads in the marker gradient were measured. The distances and corresponding densities for the standard density beads were plotted and the densities of the visible bands in the sample gradient estimated from the density gradient profile. Approximately 1-mL fractions were collected from each gradient as previously described.[20] Because Percoll interferes with some assays (β-glucuronidase,[20] polyacrylamide gel electrophoresis in sodium dodecyl sulfate [SDS-PAGE], and electron microscopy[21]), it was depleted from a portion of each fraction by ultracentrifugation in an Airfuge (Beckman) at 180,000 g for 10 minutes. As previously shown,[20] the density of Percoll in any given fraction must be greater than that of the biologic material to prevent trapping of the subcellular organelles. For that reason centrifuge tubes were balanced with dense Percoll (ρ = 1.122). After centrifugation, the biologic material was pelleted atop a dense layer of Percoll.

Enzyme assays. MPO activity was determined spectrophotometrically as the oxidation of *o*-dianisidine.[22] The change in absorption at 460 nm was measured and international units of MPO activity calculated as 2.655×10^{-4} times the rate of change in absorption at 460 nm[23] and data are expressed as international units per milliliter. Elastase activity was measured by the digestion of tritiated elastin as described by Stone et al[24] using purified human neutrophil elastase (HNE) as a standard; ^{3}H-elastin and purified elastase were the generous gifts of Dr P.J. Stone, Boston University, and data are presented as cpm/h/mL. We established that 3 μg of purified HNE solubilizes 2,417 ± 436 cpm/h (n = 11). β-Glucuronidase activity was measured spectrophotometrically as the liberation of phenolphthalein from phenolphthalein glucuronic acid (Sigma Chemical Co, St Louis) in an acetate buffer as previously described[25] and after Percoll was removed by ultracentrifugation. Data are presented as units per milliliter where 1 unit is the quantity of β-glucuronidase that hydrolyzes 1 μmoL of substrate per minute using a millimolar extinction coefficient of 33 (mmol/L)$^{-1}$cm^{-1} for phenolphthalein.

Vitamin B_{12}–binding protein was measured as binding of ^{59}Co–vitamin B_{12} (Amersham Corp, Arlington Heights, Ill) as described by Gottlieb et al.[26] Cytochrome c oxidase was measured spectrophotometrically as oxidation of cytochrome c in the manner described by Cooperstein and Lazarow.[27] Cytochrome c (Sigma) was reduced with sodium dithionite prior to the assay. Alkaline phosphatase activity was measured spectrophotometrically as the liberation of *p*-nitrophenol from *p*-nitrophenylphosphate in a sodium barbital buffer (pH 10.5) as described by DeChatelet and Cooper.[28] Data are presented as U/mL where 1 unit is the amount of alkaline phosphatase that hydrolyzes 1 μmol of *p*-nitrophenylphosphate per minute, using a millimolar extinction coefficient of 18.6 (mmol/L)$^{-1}$cm^{-1} for *p*-nitrophenol. For samples containing Percoll, an identical sample without *p*-nitrophenylphosphate was run in parallel to quantitate the turbidity at 410 nm produced by Percoll. This value was subtracted from that obtained with substrate. Sulfatase C activity was measured fluorometrically as the release of 4-methylumbelliferone from 4-methylumbelliferyl sulfate (Sigma) as previously described.[29] Data are presented as units per milliliter where 1 unit is the amount of sulfatase C that produces 1 μmol of 4-methylumbelliferyl sulfate. Galactosyl transferase activity was determined by the method of Beaufay et al[29] as the transfer of uridine diphosphate (UDP)-^{14}C-galactose to ovalbumin. The labeled UDP-galactose (Amersham) had a specific activity of 337 mCi/mmol, and approximately 0.2 μCi (590 picomoles or 6.5×10^{5} cpm) were added per reaction. Data are presented as cpm/mL. Protein was measured according to the method of Lowry et al.[30]

Spectroscopy. Absorption spectra were measured from 400 to 600 nm, using a Perkin-Elmer (Norwalk, Conn) 320 dual-beam spectrophotometer equipped with a head-on photomultiplier tube. Portions of the fractions were divided into two cuvettes, a background correction was run, and dithionite was added to the sample cuvette. The reduced minus oxidized spectrum was run and examined for peaks characteristic of cytochrome b (559, 529, 428 nm). Triton X-100 (0.1%) (Sigma, St Louis) was added to both cuvettes and the scan repeated to visualize the characteristic MPO peaks at 472 and 428 nm. As reported for PMNs,[20] HL-60 granules separated on Percoll gradients displayed the MPO spectrum only when detergent was added (ie, the granule membrane restricted passage of dithionite). The amount of MPO was quantitated using an extinction coefficient of 75 (mmol/L)$^{-1}$cm^{-1} at 472 nm,[31] and cytochrome b was quantitated using an extinction coefficient of 21.6 (mmol/L)$^{-1}$cm^{-1} at 559 nm.[32] The limit of spectroscopic detection of MPO in our hands is approximately 0.050 μmol/L.

PAGE. Percoll-depleted fractions were solubilized in gel sample buffer (62.5 mmol/L Tris-HCl, 190 mmol/L NaCL, 6 mmol/L EDTA, 2.3% SDS, 2.5% Triton X-100, 5% 2-mercaptoethanol, pH 6.8) and placed in a boiling water bath for two minutes. Bromophenol blue in 50% glycerol was added to samples prior to electrophoresis. Samples were electrophoresed into a 10% acrylamide resolving gel with a 3.5% acrylamide stacking gel. Electrophoresis was carried out overnight at 40 V (constant voltage) and voltage increased the following morning until the dye front had migrated 10.5 cm into the resolving gel.

Immunoautoradiographic detection of MPO peptides. Proteins were electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) according to the technique of Towbin et al,[33] blotting for 1.75 hours at 35 V in a Tris-glycine aqueous methanol buffer (0.025 mol/L Tris base, 0.192 mol/L glycine, 20% methanol, pH 8.3). Peptides immunochemically related to MPO were identified using anti-MPO rabbit antiserum according to a method previously described.[34] The antiserum was raised to highly purified human MPO (A_{430}/A_{280} = 0.82[35]) and has been shown to provide a sensitive and specific means for detection of MPO-related peptides.[34] The nitrocellulose paper was placed in Burridge buffer (50 mmol/L Tris base, 150 mmol/L NaCl, 0.005% NaN_3, pH 8.7) containing 3% bovine serum albumin (BSA, radioimmunoassay grade, Sigma) at 4 °C overnight. The paper was then placed in anti-MPO antiserum (1:40 in 3% BSA in Burridge buffer) for two hours at room temperature. The paper was washed extensively with multiple changes of Burridge buffer for a period of one to two hours and then placed in 50 mL of 3% BSA in Burridge buffer containing ^{125}I–protein A (1 to 3×10^{5} cpm/mL) for one hour at room temperature. The paper was extensively washed for several hours with multiple changes of Burridge buffer, left overnight in the same buffer, and again washed well the next morning. The paper was dried and autoradiographed using Kodak XAR-5 x-ray film (Eastman Kodak Co, Rochester, NY) and a fluorescence-intensifying screen. The gel was fixed and stained with R-250 Coomassie brilliant blue in 50% methanol–10% acetic acid. Mol wts were calculated from plots of R_f *v* log mol wt, using myosin (205 kilodaltons [kDa]), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) as standards (Sigma).

Electron microscopy. Percoll was removed from samples by ultracentrifugation prior to electron microscopy. Samples were fixed overnight in suspension in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.2, then pelleted in microfuge tubes. Pellets were washed with cacodylate buffer, then postfixed in 1% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer for 45 minutes. Samples were washed twice with cacodylate buffer and dehydrated by graded ethanol series and propylene oxide[36] followed by embedment in Spurr's low viscosity medium.[37] Polymerization was carried out in a vacuum oven at 70 °C for eight hours. Resultant blocks were sectioned on a Ultracut E ultramicrotome (Reichert, Buffalo). Glass knives were used for sectioning, since any residual Percoll may damage diamond knives.[21] Sections were placed on 200-mesh copper grids, stained with 5% uranyl acetate for ten minutes, washed, counterstained with lead citrate for eight minutes, and washed again. Specimens were viewed and photographed in a H-600 electron microscope (Hitachi, Mountain View, Calif) at an acceleration voltage of 50 kV in a magnification range of 5,000 to 17,000.

RESULTS

In contrast to PMNs, HL-60 cells actively synthesize protein. To identify the subcellular components of HL-60 cells involved in protein synthesis, a Percoll gradient was generated to separate these elements from lysosomes. The density profile of the gradient was determined by using colored marker beads of known density and measuring the distance of the colored bands separated in the gradient from the bottom of the tube (data not shown). When the supernatant from the low-speed centrifugation of the cavitate (S_1) was separated on the preformed gradient of Percoll, five bands were seen (indicated in the text by numbers 1 to 5), although one band (no. 4) was visible only when the gradient was heavily loaded. The resultant bands segregated into a single high-density band and four less dense bands, the latter bands visibly distinct from each other and easily aspirated from the gradient using a Pasteur pipette.

After cavitation most of the enzyme activity was present in S_1, the supernatant from low-speed centrifugation of the cavitate, indicating nearly complete disruption of the cells (Table 1). For example, 93% of the MPO activity detected in the cavitate was recovered in S_1, whereas only 7% was in P_1, the pellet of unbroken cells and nuclei. In addition, most of the enzyme activity loaded on the gradient in S_1 was recovered subsequently in fractions from the gradient. Of note, there was neither vitamin B_{12}–binding protein nor cytochrome b found in S_1, consistent with previous reports that HL-60 cells lack specific granules.[11,13,17]

Table 1. Marker Enzyme Recovery

Marker	Activity in S_1* (Percentage of Total in Cavitate)	Activity in Gradient Fractions† (% of total in S_1)
MPO	93.0	85.3
Elastase	89.0	97.0
β-Glucuronidase	72.0	75.0
Vitamin B_{12}–binding protein	none	none
Cytochrome b	none	none
Alkaline phosphatase	97.0	83.0
Sulfatase C	89.0	72.6
Galactosyl transferase	75.0	92.5

*Represents the percentage of enzyme activity present in the whole cavitate that is recovered in S_1 where $S_1 + P_1$ equals 100%.

†Represents the enzyme activity recovered from the gradient as a percentage of the activity in S_1 loaded atop the gradient.

Marker enzyme recovery is based on the results of three cavitations.

The profiles of biochemical markers in the 1-mL fractions collected from the gradient are shown in Fig 1. Fraction 1 was from the bottom of the gradient and was the most dense fraction. Since the top of the gradient was at fraction 25, activity in fractions lighter than 25 represented soluble enzyme activity (cytosol) that failed to enter the gradient. Peak activity of the lysosomal markers MPO, elastase, and β-glucuronidase, was in the dense part of the gradient, fractions 4 to 8. This corresponded to the most dense visible band, no. 1, with $\rho = 1.102$. In contrast, the enzyme markers for other organelles equilibrated in much lighter regions of the gradient. Peak activities for sulfatase C and galactosyl transferase, identified as biochemical markers for endoplasmic reticulum and Golgi apparatus, respectively,[38] cosedimented with visible bands 3 and 4 having densities of 1.039 and 1.032 g/mL, respectively. Alkaline phosphatase, a marker for plasma membrane in neutrophils,[39] had two broad peaks of activity, one in fractions 16 to 21 and one in fractions 23 to 25, the latter cosedimenting with visible band no. 5 with a density of 1.027 g/mL. Fraction 15 contained visible membrane aggregates and had substantial MPO, sulfatase C, and alkaline phosphatase activity as well as a small amount of galactosyl transferase activity at this intermediate density. This fraction also contained peak activity of cytochrome oxidase, a marker for mitochondria[27] (data not shown) and represented visible band no. 2 with a density of 1.050 g/mL. Thus it appeared that centrifugation on this gradient afforded wide separation of lysosomes from less dense organelles and provided resolution of the less dense organelles into three visible bands, cosedimenting with biochemical markers for Golgi apparatus, endoplasmic reticulum, and plasma membrane.

To confirm that lysosomes were separated from less dense organelles, we examined fractions 6, 15, and 21 by transmission electron microscopy. Electron micrographs of fraction 6 (Fig 2A), an area of the gradient rich in enzymatic activity for lysosomal markers, displayed numerous electron-dense membrane-bound lysosomelike organelles. In contrast, electron micrographs of fraction 21 (Fig 2B), a region in the gradient free of biochemical markers for lysosomes but rich in enzymatic activity of markers for other organelles, showed no lysosomal granules but had numerous membrane vesicles, presumably derived from plasma membrane, endoplasmic reticulum, and Golgi apparatus. Electron micrographs of fraction 15 (Fig 2C) contained numerous mitochondria, membrane vesicles, and lysosomes, consistent with the bio-

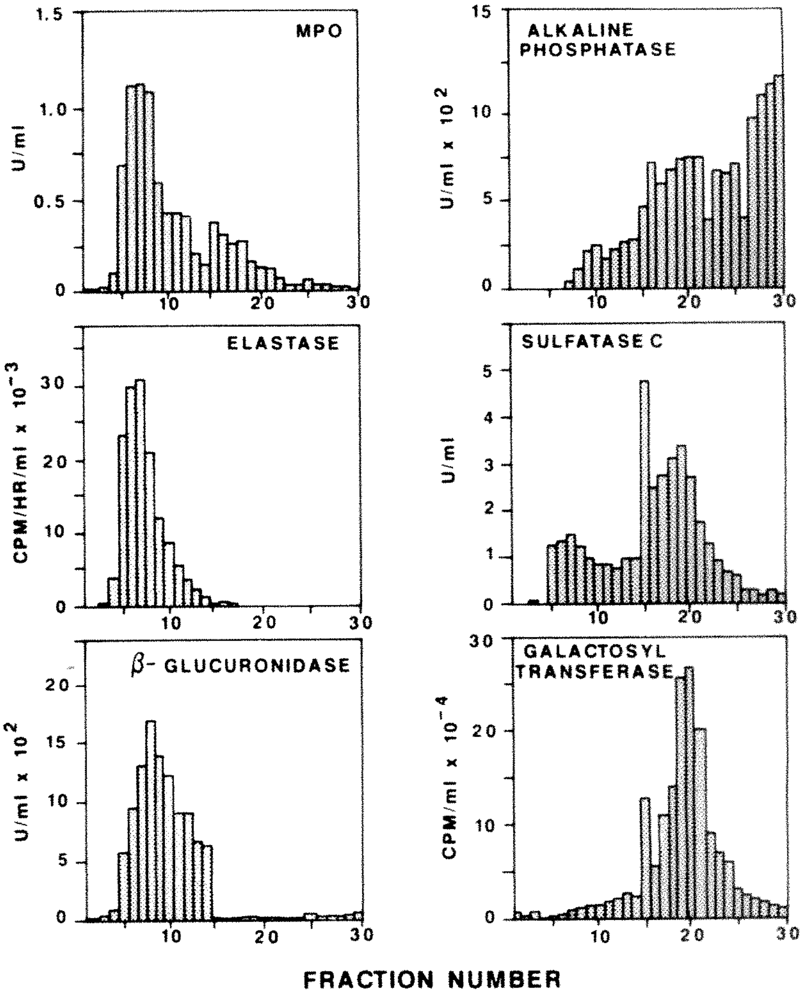

Fig 1. Profile of biochemical markers in fractions from Percoll gradient of HL-60 cells. Fraction 1 was from the bottom of the gradient and the most dense, whereas fraction 25 was at the top of the gradient; so activity in less dense fractions represented cytosolic activity that failed to enter the gradient. Peak activity of lysosomal markers (MPO, elastase, and β-glucuronidase) were in the dense part of the gradient (fractions 6 to 8) and corresponded to visible band 1 with a density of 1.102 g/mL. In contrast, biochemical markers for plasma membrane (alkaline phosphatase), endoplasmic reticulum (sulfatase C), and Golgi apparatus (galactosyl transferase) equilibrated in much less dense regions of the gradient. These less dense subcellular organelles were separated by this gradient into three visible bands, with densities of 1.039 (endoplasmic reticulum), 1.032 (Golgi apparatus), and 1.027 g/mL (plasma membrane).

chemical data suggesting this fraction contained elements of several subcellular organelles.

We have applied this analytical method to determine the subcellular location of intermediate molecular species generated during the biosynthesis of MPO (Fig 3). MPO was analyzed in three ways. First, enzymatic activity was determined in fractions from the gradient and is shown in the histogram plotting activity against fraction number. Second, MPO in selected fractions was quantitated by assessing the spectral properties of the intact heme group in mature MPO. Third, the immunochemical reactivity of the protein backbone of MPO was assessed by immunoblotting. Fractions 4 to 6, the area of the gradient where the activities of all three lysosomal enzyme markers were maximal, displayed peak MPO activity, had the highest concentration of heme-containing MPO as determined spectrally, and contained mature MPO, represented in the autoradiograph as the 59-kDa α-subunit. The 13.5-kDa β-subunit ran with the dye front in this 10% acrylamide gel and is not shown. In contrast, fractions 22 to 23, taken from the area of the gradient with peak galactosyl transferase activity, had no MPO enzymatic activity and no spectral evidence of MPO heme, but contained the 89-kDa precursor of MPO as the predominant species seen in the autoradiograph. Fractions 15 and 16 had some MPO activity, trace spectral evidence of MPO, and both mature and pro-MPO in the autoradiograph. These data are consistent with the hypothesis that pro-MPO is present in the Golgi apparatus but is devoid of enzymatic activity and without a heme group, at least within the limits of the detection assays used. Moreover, they suggest that during processing there is concomitant insertion of heme into the peptide backbone and proteolytic maturation of pro-MPO to yield enzymatically active MPO.

DISCUSSION

There is a great deal of interest in the synthesis and intracellular transport of lysosomal enzymes.[40] There is considerable evidence that nascent lysosomal enzymes are cotranslationally inserted into the lumen of endoplasmic reticulum and glycosylated at selected asparagines in the peptide backbone of the proenzyme.[41,42] During subsequent transport through the endoplasmic reticulum and Golgi apparatus, the proenzyme undergoes extensive modification of the carbohydrate side chains, including phosphorylation of mannose side chains in many cases.[43] The final transfer of the phosphorylated glycosylated proenzyme to the lysosome involves interaction with mannose-6-phosphate receptors present in the lysosomal membrane, although there is evi-

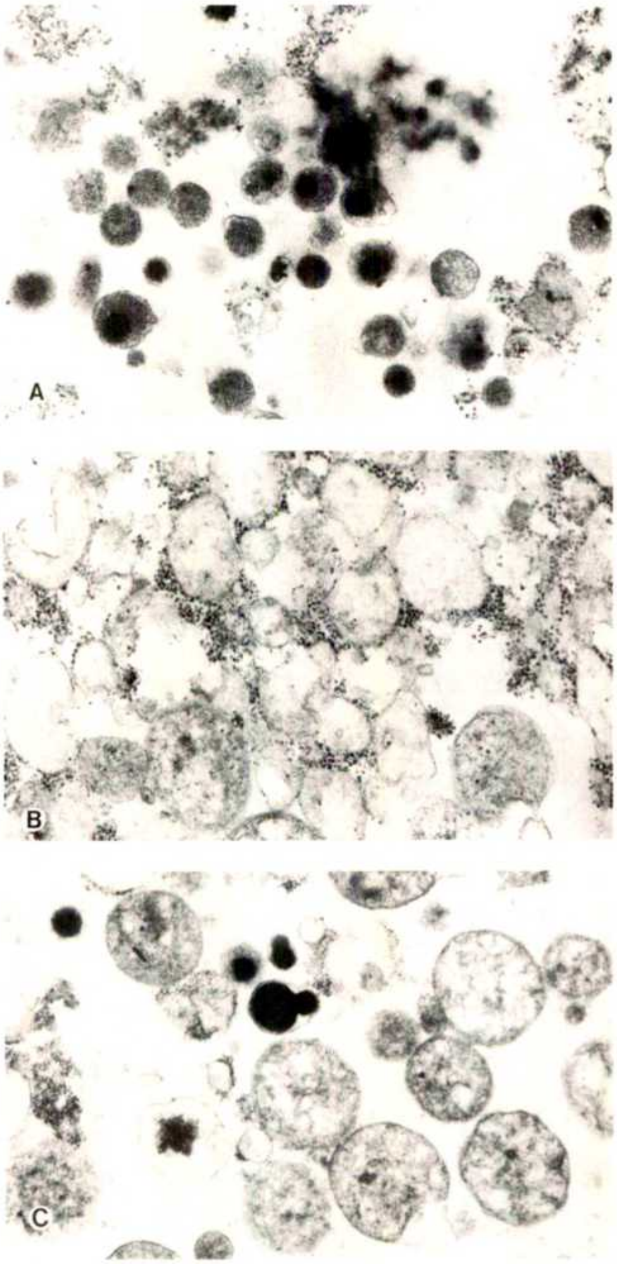

Fig 2. Electron micrographs of selected fractions from the Percoll gradient. Percoll was removed from fractions prior to glutaraldehyde fixation and electron microscopy. Fraction 6 (A) was from the region of the gradient rich in biochemical markers for lysosomes and had numerous electron-dense membrane-bound organelles consistent with lysosomes. Fraction 21 (B) was from the region of the gradient rich in biochemical markers for less dense organelles, such as Golgi apparatus and endoplasmic reticulum, but free of lysosomal enzyme activity. Electron micrographs showed numerous membrane vesicles but no lysosomes. The electron micrographs of fraction 15 (C) contained mitochondria, membrane vesicles, and lysosomes, consistent with the presence of multiple organelles as suggested by the biochemical data (original magnification for A and B is 45,000, current magnification is 22,500; original magnification for C is 37,000, current magnification is 18,500).

dence for an alternative pathway for targeting in mannose-6-phosphate receptor–deficient cells.[44] Concomitant with this final transfer is proteolytic maturation of the precursor form to a mature lysosomal enzyme.[45] In fibroblasts, for example, β-hexosaminidase undergoes final maturation from proenzyme to mature enzyme in lysosomes.[46] It is also possible that maturation occurs in a prelysosomal compartment that rapidly transfers mature enzyme to the lysosome and that cosediments with lysosomes in density gradients.[47] In those cases in which the proenzyme is not catalytically active,[45,48,49] the linking of the maturation event with transport to the lysosome provides the cell with a means of self-protection during the time such hydrolytic enzymes traverse nonlysosomal intracellular compartments. Since the precursors of some hydrolases are enzymatically active,[50] it is obvious that different mechanisms are operative in achieving the synthesis and compartmentalization of hydrolytic enzymes without cellular injury during intracellular transport.

In contrast to the fibroblast system, the study of lysosomal enzyme biosynthesis in myeloid cells has been limited by the differentiated state of peripheral blood neutrophils. The HL-60 cell line actively synthesizes lysosomal enzymes such as β-glucuronidase, elastase, and MPO and therefore provides a means for the study of the synthesis and intracellular transport of myeloid lysosomal enzymes. In addition, HL-60 cells can be induced to differentiate into functionally and morphologically more mature myeloid cells on exposure to dimethyl sulfoxide[18] or N^6,O^2-dibutyryl cyclic adenosine monophosphate,[51] thereby providing a useful system for the study of the biosynthesis of functionally important membrane proteins as was recently reported by Malech et al in a study of receptors for the formylated chemotactic peptide.[52] However, this model may be limited by virtue of the malignant lineage of the cell line.

To study the subcellular path of newly synthesized integral membrane proteins and lysosomal enzymes, we modified a previously described method for subcellular fractionation to resolve more clearly those organelles with densities of 1.020 to 1.060 g/mL. Borregaard et al[20] described a method for disruption of PMNs and separation of subcellular organelles on a gradient of Percoll. The method has major advantages over traditional methods of subcellular fractionation. Because cells are disrupted by nitrogen cavitation, organelles are not subjected to the heat or shearing forces associated with homogenization or sonication. In addition, the Percoll gradient is isotonic, in contrast to the hypertonicity of conventional sucrose gradients, thereby maintaining organelle integrity during sedimentation. Finally, isopycnic conditions are reached very quickly, allowing the determination of specific densities after short centrifugations. However, the gradient used by Borregaard et al was designed to separate azurophilic granules, specific granules, and plasma membrane vesicles and was relatively steep in the region of 1.02 to 1.10 g/mL. Since PMNs have few mitochondria and little endoplasmic reticulum or Golgi apparatus, organelles that would sediment at this density, there was no need to resolve them in a density gradient designed for the study of PMNs.

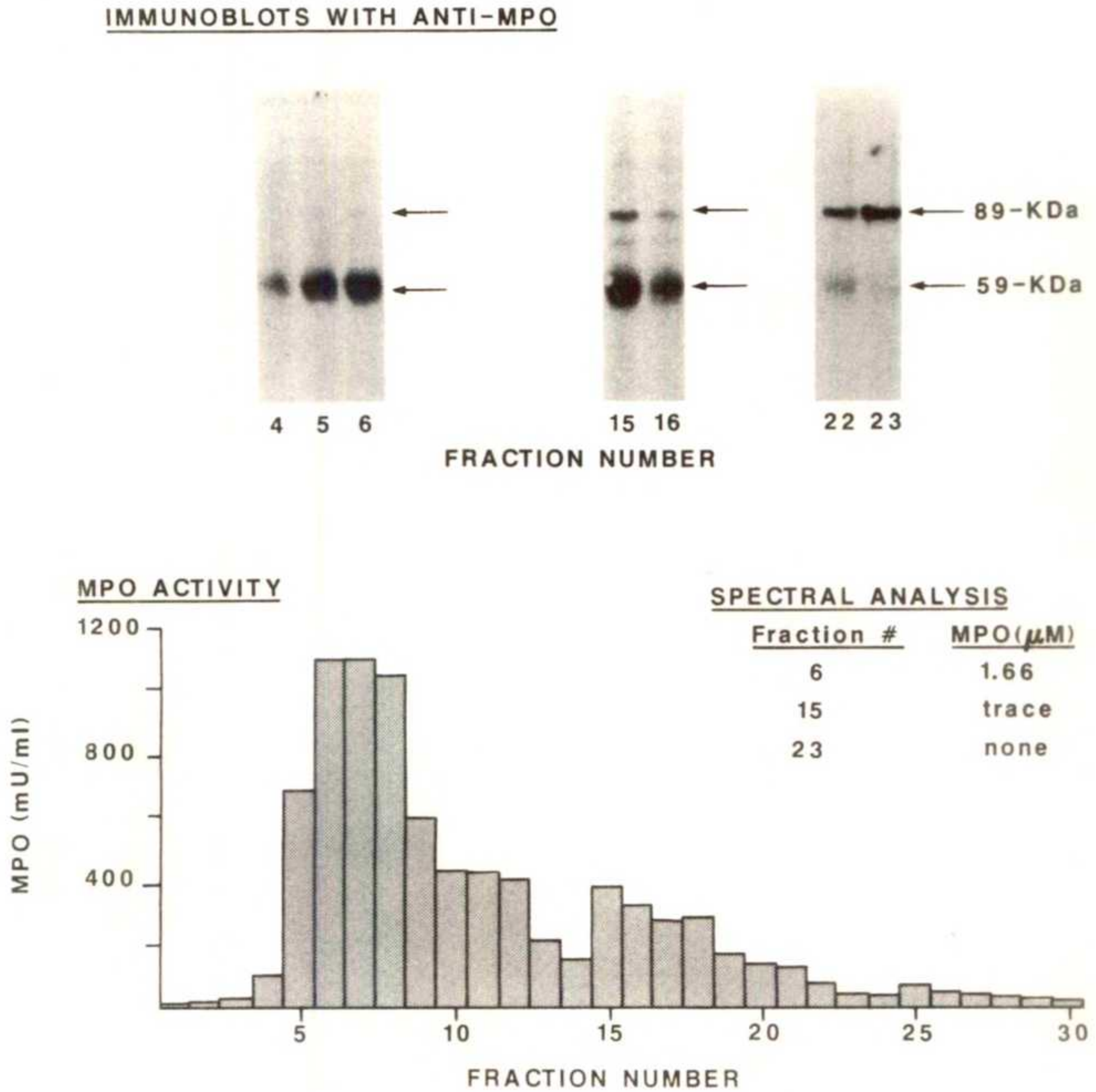

Fig 3. Subcellular localization of MPO processing. The presence of MPO species was evaluated in three distinct ways: enzymatic activity (shown in histogram) was measured using the *o*-dianisidine assay to assess peroxidative activity, the presence of a heme group was assessed spectrally using oxidation-reduction difference spectroscopy, and the MPO peptide backbone was analyzed immunochemically using electroblotting and anti-MPO antiserum. Fractions 4 to 6, the area of the gradient where the lysosomal markers cosedimented, displayed peak MPO activity, had the highest concentration of the heme-containing MPO, and contained mature MPO, identified in the autoradiograph by the 59-kDa α-subunit. In contrast, fractions 22 to 23, which cosedimented with markers for less dense organelles, lacked MPO activity and spectral evidence of heme, but contained the 89-kDa precursor of MPO as shown by immunoautoradiography. Fractions 15 and 16 contained MPO activity, trace amounts of heme-containing MPO, and immunochemical evidence of mature and precursor forms of MPO.

The modified gradient described here separated the lysosomal compartment from organelles of lower density. The high-density fractions contained azurophilic granules, whereas the low-density fractions contained endoplasmic reticulum, Golgi apparatus, and plasma membrane. These latter fractions had no activity for any lysosomal enzymes and by electron microscopy were free of lysosomes but rich in membrane vesicles. Thus, our method of subcellular fractionation allows separation of lysosomes from less dense organelles important for the synthesis and proper targeting of lysosomal enzymes.

We applied this method of subcellular fractionation to HL-60 cells to determine to what extent synthesis and transport of a myeloid lysosomal enzyme such as MPO follow the schema derived from studies of other cells. MPO is a glycoprotein containing high mannose side chains and is synthesized in vivo as an 89-kDa proenzyme and subsequently packaged into azurophilic granules as a heterodimer ($\alpha_2\beta_2$) composed of two heavy-light protomers of 59,000 and 13,500 mol wt as determined under reducing conditions in SDS-PAGE.[34] As predicted, mature MPO cosedimented with lysosomal enzyme markers whereas the 89-kDa pro-MPO cosedimented with elements of the Golgi apparatus and plasma membrane. Of note, both mature MPO and pro-MPO were found in one intermediate area of the gradient (fractions 15 and 16) where there was activity of alkaline phosphatase and sulfatase C as well as MPO. This may represent a prelysosomal compartment in which the pro-MPO undergoes proteolytic maturation. However, further studies to improve resolution of organelles at this density are needed to exclude the possibility of contamination of these fractions by soluble MPO nonspecifically adherent to endoplasmic reticulum. Within the limits of oxidative-reduction difference spectroscopy, our data suggest that pro-MPO lacked heme, whereas the mature MPO present in lysosomes contained a prominent heme group. Thus, maturation of pro-MPO appears to require insertion of a heme as well as proteolytic processing into the native MPO. Currently, studies directed at identifying the timing and subcellular location for heme incorporation are under way.

This method of subcellular fractionation of HL-60 cells provides a useful system to dissect further the processing and intracellular transport of myeloid lysosomal enzymes. Understanding these events may provide insight into defects in lysosomal enzymes in myeloid cells (eg, MPO deficiency) as well as identify mechanisms by which the normal myeloid cell sorts proteins destined for azurophilic granules from those destined for specific granules and for plasma membrane.

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Association Between Morphologic Distortion of Sickle Cells and Deoxygenation-Induced Cation Permeability Increase

By Narla Mohandas, Mary E. Rossi, and Margaret R. Clark

We hypothesized that the deoxygenation-induced increase in cation permeability of sickle cells was related to mechanical distention of the membrane by growing HbS polymer within the cell. To test this hypothesis, we determined the effect of deoxygenation on cation fluxes in sickle cells under conditions that restricted or permitted extensive growth of polymer, producing different degrees of membrane distention. Manipulation of suspending medium osmolality for density-isolated high and low mean cell hemoglobin concentration (MCHC) cells was used to regulate the extensional growth of polymer bundles and hence membrane distortion. For initially low MCHC cells, the deoxygenation-induced increase in both Na and K fluxes was markedly suppressed when the MCHC was increased by increasing the osmolality. This suppression corresponded to the inhibition of extensive morphologic cellular distortion. For initially high MCHC, ISC-rich cells, deoxygenation had minimal effect on K permeability. However, reduction of MCHC by a decrease in osmolality produced a concomitant increase in cation permeability and cellular distortion. These observations support the idea that the sickling-associated increase in membrane permeability is related to mechanical stress imposed on the membrane by bundles of HbS polymer.

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ALTHOUGH polymerization of Hb S is a cytoplasmic event, it also has effects on the function of the red cell membrane. Perhaps the most dramatic example of this is the massive increase in membrane permeability to monovalent cations, as first described by Tosteson et al.[1,2] In the 30 years since this important observation, the mechanism producing the permeability increase has not been elucidated. Because of the association between abnormal cation and volume regulation and the impaired rheology of sickle cells,[3-5] clarification of this phenomenon is of considerable interest.

On the basis of previous observations that the magnitude of the permeability increase correlated with the proportion of morphologically distorted, sickled cells,[6] we hypothesized that membrane distortion itself may be directly related to increased permeability. More specifically, we conjectured that mechanical stress on areas of the membrane that surround extended spicules of hemoglobin S polymer could produce local sites for increased cation flux. To test the relationship between morphologic distention and cation permeability, we made use of our earlier observation that the extent of sickle cell distortion was related to the hemoglobin concentration inside the cell.[7] We had shown that low MCHC discoid sickle cells assume highly spiculated shapes when deoxygenated in isotonic medium but undergo a relatively minor shape change when deoxygenated in a dehydrated state in hypertonic medium. Conversely, high MCHC irreversibly sickled cells (ISC) undergo little shape change when deoxygenated in isotonic medium, but form characteristic spiculated, sickled shapes when their MCHC is reduced by suspension in hypotonic medium. This difference in morphology may be related to the observations that the number of polymer domains formed during rapid deoxygenation (and presumably, rapid warming of chilled deoxygenated samples) varies with the concentration of HbS. Because polymerization depends on the concentration-dependent process of nucleation, low HbS concentration favors aligned polymer growth in a single or few domains, whereas high HbS concentration favors the growth of polymer in multiple, independent domains.[8,9] By comparing the magnitudes of the deoxygenation-induced change in cation permeability in these two distinct morphologic states for each cell type, we hoped to determine whether cation permeability could be correlated with membrane distention.

MATERIALS AND METHODS

After informed consent was obtained from subjects with homozygous sickle cell disease, blood samples were drawn into either heparin or acid-citrate-dextrose (ACD). Red cells were separated from plasma, resuspended in buffered saline containing potassium and glucose (135 mmol/L of NaCl, 5 mmol/L of KCl, 8.6 mmol/L of $Na_2H\ PO_4$, 1.4 mmol/L of $NaH_2\ PO_4$, 11 mmol/L glucose adjusted to pH 7.4 and 290 ± 5 mosm/kg), and were then separated to provide either a low MCHC (32 ± 0.5 g/dL), mature discoid cell population or an ISC-enriched population (39 ± 1.6 g/dL), using discontinuous Stractan density gradients as previously described.[10] For measurements of permeability, the isolated cell populations were incubated at 37 °C, 5% hematocrit, for 4.5 hours in potassium-free, phosphate-buffered saline (PBS) (5 mmol/L of Na phosphate, pH 7.4, total Na concentration, 135 mmol/L). The medium also contained 0.1 mmol/L of ouabain to suppress active transport of Na and K, 10 mmol/L of glucose, and 100 U/mL of penicillin and 100 µg/mL of streptomycin (penicillin-streptomycin, Grand Island Biological Co, Grand Island, NY) to inhibit bacterial growth. The isotonic and hypotonic media contained 27 mmol/L of sucrose to minimize hemolysis, and the hypertonic solution contained additional sucrose to attain the desired osmolality without changing the Na concentration. However, the osmolality of the hypotonic solution was adjusted by reducing the NaCl concentration. For experiments using low MCHC cells, paired samples were incubated at 290 and 550 mosm/kg, whereas ISC-rich samples were incubated at 290 and 110 mosm/kg. To determine the effect of deoxygenation, identical pairs were incubated under room air and nitrogen atmospheres. To insure a well-defined starting point for the flux measurements, the spending media for deoxy samples were first deoxygenated by bubbling humidified N_2 gas through them for 20 minutes while maintaining them at ice temperature. The cells were then added, and deoxygenation was continued by surface gassing for an additional

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ten minutes, after which the incubation vessels were sealed. Because hemoglobin S does not polymerize at 0 °C, we were thus able to deoxygenate the cells first and then rapidly initiate polymerization by transferring the cell suspensions to a 37 °C water bath. The oxygenated samples were carried through the temperature changes in parallel with the deoxygenated samples. Initial samples were removed from the incubation mixtures before they were warmed to 37 °C, and three additional samples were removed anaerobically at 90-minute intervals. These samples were separated by centrifugation, and the red cells were washed three times in isotonic Tris-buffered (10 mmol/L, pH 7.4 at 0 °C) magnesium chloride. Both the initial supernatant incubation medium and washed cells were assayed for Na and K by flame photometry. Hemoglobin measurements on the incubation cell suspensions, together with hemoglobin and hematocrit determinations on the initial cell samples at incubation tonicities, were used to determine changes in intracellular cation concentration and content. Morphologic examination of cells removed anaerobically from the incubation vessels confirmed our prior observations (Fig 1) concerning the effect of MCHC on the morphology of deoxygenated sickle cells. The isotonic, initially discoid cells and the hypotonic ISC showed well-defined spicule formation, whereas the hypertonic discoid cells and isotonic ISC did not form extensive spicules.

Data analysis Estimates of potassium efflux were obtained from measurements of potassium in both the incubation medium and the cells. Measurement of hemoglobin concentrations in the suspending medium throughout the experiment showed that hemolysis in the oxygenated samples was only 1% to 2%, and rose to 4% to 5% in the deoxygenated samples. No significant effects of changing the medium tonicity were observed. This contribution to the medium K concentration was insignificant in comparison to the total cation changes, so no correction for hemolysis was applied. Using the cation data, we calculated first-order rate constants for potassium efflux as described elsewhere.[10] To evaluate the effect of MCHC on the deoxygenation-induced increase in Na permeability, we used linear regression analysis to determine the increase in intracellular content during the experiment. This was done only for the low-density cell population, since the initially higher Na concentrations of the high-density cells reduced the sensitivity of the assay. The increases in Na content were then expressed on the basis of cell Hb content, to take into account shrinkage of the cells in the hypertonic medium. To determine the effect of suspending medium tonicity (and concomitant morphologic differences) on both K rate constants and Na uptake, we determined the differences obtained by incubating cells at a given tonicity under an N_2 or an O_2 atmosphere. These deoxygenation-induced differences were then separately compared for low MCHC and ISC-rich cell populations at the two incubation tonicities, using a *t* test[11] for independent samples.

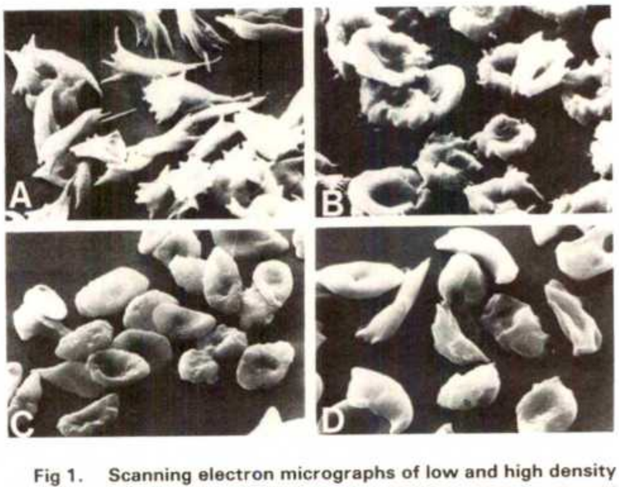

Fig 1. Scanning electron micrographs of low and high density sickle cells deoxygenated at different MCHCs: initially discoid sickle cells, deoxygenated at (A) low and (B) high MCHC; ISC-rich sickle cells deoxygenated at (C) high and (D) low MCHC.

RESULTS

Low MCHC sickle cells, when incubated in isotonic medium, showed the expected marked increase in ouabain-resistant K loss when deoxygenated (Fig 2). Increase of suspending medium tonicity to 550 mosm/kg caused an increase in intracellular K concentration, because of osmotically induced cell shrinkage, which resulted in an accelerated loss of K, even under oxygen. When these dehydrated cells were deoxygenated, K loss was further augmented. Under the conditions of these experiments, in which extracellular K concentration was near zero, the rate of K efflux was expected to depend on the intracellular K concentration. To test this expectation, we examined the relationship between the initial efflux rates and intracellular K concentration. Separate linear relationships were observed for oxygenated and deoxygenated cells (data not shown), suggesting that, as for ouabain-resistant K efflux from normal cells and K influx into deoxygenated sickle cells at extracellular K concentrations >5 mmol/L,[1] K efflux from both oxygenated and deoxygenated sickle cells has a first-order dependence on intracellular K concentration. Therefore, we calculated first-order rate constants to facilitate comparison of membrane

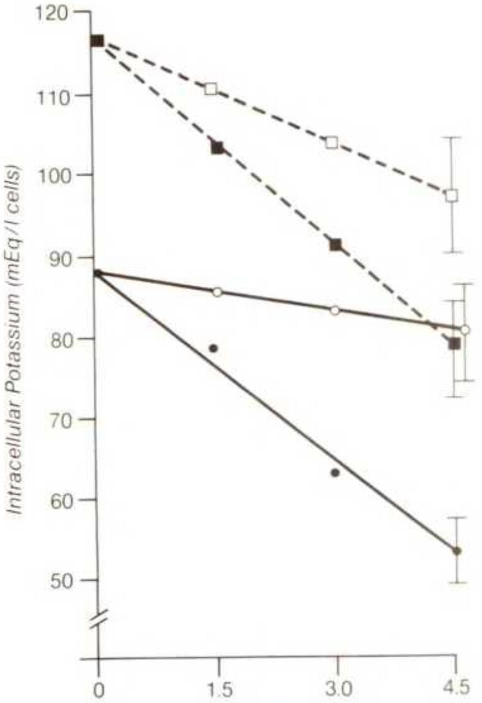

Fig 2. Intracellular K concentration of low MCHC (32 ± 0.5 g/dL) sickle cells during incubation under N_2 or O_2 at 290 and 550 mosm/kg. Deoxygenated samples (solid symbols); oxygenated samples (open symbols); 550 mosm/kg medium (squares); 290 mosm/kg (circles). Bars indicate SEM.

Table 1. Effect of Deoxygenation on First-Order Rate Constants for K Efflux From Low and High MCHC Sickle Cells

Cell Sample	N	MCHC (g/dL)	k^0_{oxy}	k^0_{deoxy}	$k^0_{deoxy}-k^0_{oxy}$
Low MCHC cells					
290 mosm/kg	6	32.1 (.5)	.0225 (.0072)	.121 (.015)	.0985
550 mosm/kg	5	41.0 (.6)	.0406 (.0041)	.0824 (.039)	.0418*
High MCHC cells					
290 mosm/kg	6	38.9 (1.6)	.0540 (.0062)	.0783 (.0081)	.0243
110 mosm/kg	3	25.3 (3.2)	.113 (.020)	.232 (.057)	.119†

First-order rate constants were calculated from the measured ion fluxes and intracellular K concentrations. Values given are the mean for N experiments. SEM is given in parentheses. By *t* test for independent samples, $^*P < 0.001$ and $^†P < 0.02$.

permeability in these cells that contained varying K concentrations.[12] As shown in Table 1, the rate constant for oxygenated sickle cells was moderately increased when the cells underwent shrinkage in hypertonic medium. However, when the cells were deoxygenated, the K efflux rate constant was actually lower for the hypertonic cells than for the isotonic ones. The net effect of deoxygenation on the rate constant for isotonic, normovolemic cells was more than twice that for the hypertonic, shrunken cells, and this difference was highly significant, with a *P* value of .001 (Table 1).

The effect of cell shrinkage on the deoxygenation-induced cation leak was even more striking when the net uptake of Na was examined. Changes in intracellular Na were inversely related to those in intracellular K (Fig 3). As shown in Table 2, the net increases in Na content were only slightly larger for oxygenated cells in isotonic medium than in hypertonic medium. However, the cells that were deoxygenated in isotonic medium showed a markedly larger uptake of Na than did the cells shrunken in hypertonic medium (nearly fourfold). Again, the increase in Na uptake that was produced by deoxygenation was strongly inhibited by cell shrinkage to a highly significant extent (Table 2). Because the tonicity of the suspending medium was increased by adding sucrose, the shrunken cells would have been subject to a smaller transmembrane gradient of Na, and an increased intracellular chloride concentration would have been associated with a less negative membrane potential. Both of these effects, which would increase Na efflux, would be expected to decrease net Na uptake at an equal proportion in the oxygenated and deoxygenated cells. Thus, the observation that the effect of increased tonicity in suppressing Na uptake was much greater when the cells were deoxygenated suggests that this suppression was not simply due to an osmotically induced increase in Na efflux. The parallel reduction in K efflux from shrunken deoxygenated cells also provides support for an independent effect of cell shrinkage. When the cells were examined microscopically, it was found that the suppression of the deoxygenation-induced increase in cation permeability corresponded to the absence of highly spiculated and distorted cells in the hypertonic deoxygenated cell suspensions. Cells deoxygenated in isotonic medium showed

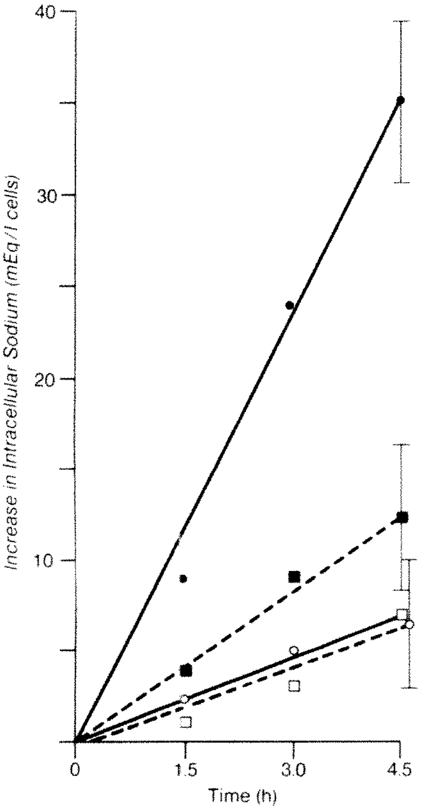

Fig 3. Changes in intracellular Na concentration of low MCHC (32 ± 0.5 g/dL) sickle cells during incubation under N_2 or O_2 at 290 and 550 mosm/kg. Deoxygenated samples (solid symbols); oxygenated samples (open symbols); 550 mosm/kg (squares); 290 mosm/kg (circles). Bars indicate SEM.

Table 2. Effect of Osmotically Induced Shrinkage on Net Increase in Na Content of Low-Density Sickle Cells Incubated Under Nitrogen or Room Air

	Isotonic Cells	Hypertonic Cells
MCHC (g/dL)	32.1 (.5)	41.0 (.6)
Initial cell [Na]		
mEq/l cell	22.1 (1.5)	28.2 (2.0)
mEq/10 g Hb	.688 (.047)	.688 (.049)
Net uptake of Na-mEq/10 g Hb		
Oxy	.046 (.014)	.037 (.016)
Deoxy	.252 (.036)	.066 (.012)
Deoxy-oxy	.175	.029*

Paired samples of low-density sickle cells were incubated under nitrogen or room air at 290 or 550 mosm/kg. Effects of deoxygenation in causing increased Na uptake were compared for isotonic and hypertonic samples, using a *t* test for independent samples; a $^*P$ value of $< .001$ was found.

Net increase in Na content was expressed on the basis of cell Hb to take cell shrinkage into account.

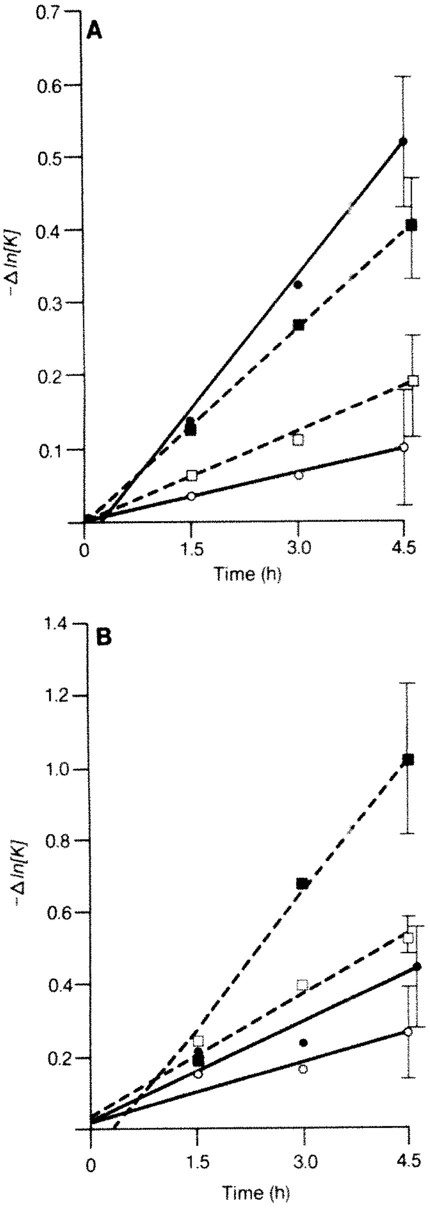

Fig 4. Changes in the natural log of intracellular K concentration of low (32 ± 0.5 g/dL) and high (39 ± 1.6 g/dL) MCHC sickle cells during incubation under N_2 or O_2 at various osmolalities. (A) Low MCHC cells. Deoxygenated samples (solid symbols); oxygenated samples (open symbols); 550 mosm/kg (squares); 290 mosm/kg (circles) (B) High MCHC cells; deoxygenated samples (solid symbols); oxygenated samples (open symbols); 110 mosm/kg (squares); 290 mosm/kg (circles). Bars indicate SEM.

the characteristic sickled shape, with distinct spicule formation.

In contrast to the effects of deoxygenation on low MCHC cells, deoxygenation of ISC in isotonic medium caused only a modest increase in K efflux (Table 1). When deoxygenation was carried out in hypotonic medium, the cells showed a greater increase in K loss (Fig 4). The difference in deoxygenation-induced increase in rate constant associated with the tonicity change was significant with a P value of .02 (Table 1). In accord with results for the low MCHC cells, the larger effect of deoxygenation was again associated with distortion of the deoxygenated cells into spiculated, characteristically sickled shape.

DISCUSSION

In the present studies of sickle cells, a consistent relationship was found between the augmentation in cation permeability induced by deoxygenation and the distortion of the membrane by extended spicules of hemoglobin S polymer. When formation of these long spicules was inhibited because of high MCHC and high cytoplasmic viscosity,[7-9] deoxygenation was much less effective in increasing membrane permeability. It might be argued that the experimental manipulations used to vary MCHC in any one set of experiments could have produced the differences in ion flux that were associated with these differences in cell morphology. However, the fact that we were able to show this in low MCHC cell populations by using hypertonic medium to inhibit the deoxygenation-induced leaks for both K and Na, and in high MCHC cells by using hypotonic medium to unmask the K leak, provides substantial support for the hypothesis that membrane distortion is necessary for the sickling-induced cation leak. Thus, although no one of the experiments would be sufficient to infer an association between the extent of cell distortion and increased membrane permeability, the consistency of this association under different conditions is significant. Furthermore, polymer formation alone cannot explain the deoxygenation-induced increase in membrane permeability, since the less deformed cells with high MCHC would have contained more polymer than the spiculated low MCHC cells.

Other investigators have suggested that mechanical stress imposed upon the sickle cell membrane by polymer spicules can disrupt other aspects of normal membrane function, such as maintenance of bilayer phospholipid asymmetry.[13] In other studies, manipulation of suspending medium tonicity, similar to the approach used in the present study, showed a correspondence between the extent of membrane distortion and increase of lipid translocation, as well as loss of phospholipid asymmetry.[14,15] Taken together, these observations support the hypothesis that the effects of HbS polymerization on both lipid organization and cation permeability are augmented or perhaps mediated by mechanical distention of the membrane.

Previously, it was noted that ISC-rich cell populations exhibited minimal effects of deoxygenation on disturbances of phospholipid organization.[16] The present study shows that deoxygenation-induced cation permeability changes are also substantially reduced in the ISC-rich population. We attribute the damping of both of these effects to the inability of the highly concentrated hemoglobin within ISC to elaborate long spicules of hemoglobin polymer that mechanically stress the membrane. Recently, Embury et al reported that thalassemic sickle cells showed a smaller change in cation permeability upon deoxygenation than did nonthalassemic sickle cells.[17] Subsequently, it was found that these cells also have an excess surface area-to-volume ratio.[18] We speculate that this redundant surface area can accommodate polymer formation with less membrane distention and may explain the smaller cation leak.

It has frequently been suggested that the deoxygenation-induced cation leak of sickle cells may play a role in the pathophysiology of the disease. The results of the present study suggest that it may not be necessary to inhibit hemoglobin polymerization completely, but that agents that limit large-scale growth of polymers may reduce the cation leak and hence cellular dehydration.

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Detection of In Vitro and In Vivo Cleavage of High Molecular Weight Kininogen in Human Plasma by Immunoblotting With Monoclonal Antibodies

By Mauro Berrettini, Bernhard Lämmle, Terry White, Mary Jo Heeb, Hans P. Schwarz, Bruce Zuraw, John Curd, and John H. Griffin

Purified human high-mol-wt kininogen (HMWK), the cofactor of the contact phase of blood coagulation, migrated as a single band (~110,000 mol wt) in a continuous buffer sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), but appeared as two separated bands (~120,000 and 105,000 mol wt) when analyzed in a discontinuous buffer SDS-PAGE system. After elution from SDS polyacrylamide gels, each of the two bands showed coagulant activity. Six murine monoclonal antibodies (Mabs) against HMWK were produced and purified. In immunoblotting studies, three Mabs bound to the isolated alkylated heavy chain and one to the alkylated light chain of HMWK, whereas the remaining two bound only to the single-chain or unreduced two-chain molecule. None of the Mabs inhibited the clotting activity of HMWK or its binding to kaolin. Two of the Mabs, one directed against the light chain and one against the heavy chain, were used as specific probes to study HMWK in plasma samples using an immunoblotting technique. The anti-light chain Mab identified two distinct bands (~120,000 and ~105,000 mol wt) in normal human plasma, but not in plasma from patients with hereditary HMWK deficiency. The anti-heavy chain Mab detected two additional bands (~60,000 and ~54,000 mol wt) corresponding to low-mol-wt kininogen (LMWK) in normal plasma. A sensitive and specific quantitative immunoblotting assay of HMWK antigen in plasma was developed. Moreover, the immunoblotting technique with the anti-light chain Mab was used to detect the cleavage of HMWK in plasma samples after in vitro or in vivo activation of the contact system. The anti-light chain Mab demonstrated in vivo activation and cleavage of HMWK during an angioedema attack in a patient with hereditary angioedema and C1-inhibitor deficiency.

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A POTENT vasoactive peptide, bradykinin, is liberated in plasma from two precursor proteins, high- and low-mol wt kininogens (HMWK and LMWK), by the action of specific enzymes termed kallikreins.[1,2] Human HMWK and LMWK are single-chain glycoproteins, with mol wts of ~110,000 to 120,000 and 50,000 to 70,000, respectively. Each protein consists of three domains: an amino terminal heavy chain, a bradykinin moiety, and a carboxyl terminal light chain.[3,4] Based on cloning and sequence analysis of cDNAs for human high- and low-mol-wt prekininogens, a complete identity in the amino acid sequence has been determined for a large portion of the two molecules, including the heavy chain, the bradykinin sequence, and the first part of the carboxyl terminal region; the two proteins are transcribed from the same gene.[5] The structural homology accounts for the recently discovered thiol protease inhibitory activity common to both proteins.[6-8]

The unique structure of the light chain of HMWK, however, provides this protein with the function of accelerating the reactions of the contact phase of blood coagulation, whereby HMWK acts as a nonenzymatic cofactor by facilitating the proteolytic activations of factor XII, prekallikrein, and factor XI on a negatively charged surface.[9,10] One of the activation products, kallikrein, potently cleaves HMWK at two specific sites to liberate bradykinin and to form a kinin-free protein.[11-13] The cleavage occurs within a critical disulfide bridge so that single-chain HMWK is converted to a two-chain molecule, in which a heavy chain of ~66,000 mol wt is disulfide-linked to a light chain of ~55,000 mol wt. The light chain is further cleaved by kallikrein to a smaller chain of 45,000 mol wt.[13]

The pathophysiologic role of plasma HMWK still remains largely unclear. The hereditary deficiency of this protein is not associated with a bleeding disorder.[14] Because bradykinin possesses a number of actions potentially involved in inflammation reactions, including vasodilation, increase in vascular permeability, and pain generation, a role for HMWK and the other proteins of the contact activation system in the pathogenesis of several pathological conditions has been suggested.[14] Evidence of HMWK cleavage in vivo, however, has been indirect only and has involved functional and/or immunological measurements of plasma levels of the protein or its released product, bradykinin.

We have prepared a series of monoclonal antibodies (Mabs) against HMWK and describe here their use as specific qualitative and quantitative probes for HMWK in plasma samples subjected to immunoblotting.

MATERIALS AND METHODS

Materials

The following reagents were obtained from sources as indicated: rabbit antibodies against the Fc region of mouse immunoglobulins (Cappel Lab, Westchester, Pa); rabbit antibodies against subtypes of mouse heavy and light chains (Miles Lab, Eckhart, Ind); Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% newborn calf serum, 10% NCTC (National Cancer Tissue Culture) 109 medium, pyruvate (Microbiological Associates, Bethesda, Md);

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Hepes, glutamine, penicillin, streptomycin (GIBCO, Grand Island, NY); dithiothreitol (DTT, Boeringer Mannheim, FRG); 0.45 μm nitrocellulose membranes (Bio Rad, Richmond, Calif); Na-^{125}I (Amersham International, England); diethyaminoethanol (DEAE)-Sephadex A50, SP-Sephadex C-50, Protein A-Sepharose CL-4B, mol wt standards (Pharmacia Fine Chemicals Inc, Piscataway, NJ); all other reagents were the best grade available.

HMWK-deficient plasma was purchased from George King Bio-Medical Inc, Overland Park, Kan. Plasma samples from a patient with hereditary C1-inhibitor deficiency were collected in 3.8% sodium-citrate using plastic tubes during and after an attack of angioedema that did not require any specific therapy. A normal human plasma (NHP) pool or individual plasma samples were prepared in our laboratory by collecting blood from 17 normal male subjects according to standard procedures. Informed consent was obtained from normal subjects and patients. Blood was usually anticoagulated with 3.8% sodium-citrate (9:1/vol:vol) and, in some experiments, blood contained benzamidine (8 mmol/L) or soybean trypsin inhibitor (SBTI, 100 μg/mL) or leupeptin (0.25 mmol/L). Plasma from EDTA (10 mmol/L) or heparin (10 IU/mL) anticoagulated blood, with or without inhibitors, was used where indicated.

General Methods

Sodium dodecyl sulfate (0.1%) polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels (1.5 mm thick) according to the methods of either Weber et al[15] or Laemmli.[16] Samples (purified proteins or plasma) were mixed with appropriate sample buffer[15,16] containing 2.5% SDS, heated for five minutes at 100 °C, and applied to the gels. After electrophoresis, gels were immediately used for immunoblotting (see below) or stained with Coomassie Brilliant Blue R250. Apparent mol wts were determined by running standard proteins of known mol wt (range 14,000 to 94,000 mol wt) on the gel. In some experiments, unstained duplicate gels were sectioned into 1-mm segments, and each slice was suspended in 0.2 mL of 0.01 mol/L of Tris, pH 7.4 containing 0.14 mol/L of NaCl and 1 mg/mL of bovine serum albumin (TBS-BSA) overnight at 4 °C. After extensive dialysis against TBS-BSA and protein concentration measurements, eluates of selected slices and control eluates were assayed for HMWK clotting activity and used for cleavage experiments by human plasma kallikrein, as described below.

Radioiodination of HMWK was performed by the chloramine T method.[17] ^{125}I-HMWK had a specific radioactivity of 4.5 μCi/μg protein and retained its original clotting activity after the radiolabeling procedure. The same method was used to obtain ^{125}I-rabbit anti-mouse IgG with a specific radioactivity of ~1 to 3 μCi/μg.

The procoagulant activity of HMWK was measured by a one-stage assay, using a congenital deficient plasma as a substrate and an automated coagulometer (COAG-A-MATE 2001, General Diagnostics, Morris Plains, NJ) to record the activated partial thromboplastin time.

Protein determination and immunodiffusion were performed according to Lowry et al[18] and Ouchterlony and Nilsson,[19] respectively.

Protein Purification

HMWK was purified from human plasma as previously described.[12] Human plasma kallikrein, produced by proteolytic activation of prekallikrein with β-factor XII$_a$,[20] was used to cleave HMWK and to prepare the two-chain, kinin-free molecule, from which the purified and alkylated heavy and light chains were prepared using SP-Sephadex C-50 chromatography.[12]

Production of Mabs

Murine Mabs to HMWK were prepared by a modification of the method of Köhler and Milstein.[21] In brief, female BALB/c mice were each injected intraperitoneally with 50 μg of purified HMWK mixed with complete Freund's adjuvant for the first injection (day 1) and thereafter with antigen in incomplete Freund's adjuvant (day 16). Three days prior to cell fusion (day 35), 50 μg of HMWK was injected intravenously (IV). Spleen cells (8.6×10^8) from three immunized mice were fused with P3X63aG8653 murine myeloma cells (1.7×10^8) using 30% (wt/vol) polyethylene glycol-1000. After two days of 4×10^{-7} mol/L aminopterin treatment, the cells were seeded into 96-well microtiter plates at 1.5×10^4 cells per well. Fused cells were cultured in DMEM supplemented with 20% newborn calf serum, 10% NCTC 109 medium, oxalacetate (1 mmol/L), pyruvate (0.45 mmol/L), glutamine (2 mmol/L), penicillin and streptomycin, Hepes (20 mmol/L), hypoxanthine (1×10^{-4} mol/L), and thymidine (3×10^{-5} mol/L). After 15 days, supernatants were tested for reactivity against purified HMWK by a solid-phase radioimmunoassay (SPRIA). For these assays, each well of 96-well plastic microtiter plates was coated, overnight at 4 °C, with 0.01 mol/L phosphate, pH 7.4, 0.14 mol/L of NaCl (PBS) containing 100 ng of single-chain HMWK, two-chain HMWK, the isolated alkylated light chain, or the isolated alkylated heavy chain. The plates were washed three times with PBS and patted dry, and 200 μL of blocking buffer (3% BSA, 0.02% NaN_3 in PBS) was added and incubated for one hour at 37 °C. Plates were again washed three times with PBS; then hybridoma culture supernatants were added in a volume of 50 μL per well and incubated for two hours at 37 °C. Plates were washed three times with PBS; then 50 μL of ^{125}I-labeled rabbit anti-mouse IgG (200,000 cpm per well in blocking buffer) was added to each well and incubated at 37 °C for two hours. Plates were again washed three times with PBS, and the radioactivity of each cut plastic well was counted, using a Micromedic 4/600 automatic γ counter. Ten of 193 positive hybridomas were selected for cloning by limiting dilution. Thymus cells (5×10^5/mL) from BALB/c mice were added to the wells as a feeder layer during cloning. Of 526 clones that were again found positive for antibodies against HMWK by the SPRIA, 8 were recloned to ensure monoclonality; 212 clones from the second cloning were again screened by SPRIA and six cell lines were selected for injection (1×10^6 cells per animal) into the peritoneal cavity of pristane-primed BALB/c mice. The ascitic fluid obtained from each mouse was tested by a SPRIA and by a dot-immunobinding assay.[22] For the latter test, various proteins including purified single-chain or two-chain HMWK, the purified heavy and light chains, or control proteins (Hageman factor, prekallikrein, factor XI, albumin) were directly spotted onto a nitrocellulose membrane. The immunodetection of the antigen was then accomplished by a procedure identical to that described below under "Immunoblotting." Of 36 ascitic fluids, six were further processed to purify the immunoglobulin fractions by Protein A-Sepharose CL-4B affinity chromatography.[23] A purified murine Mab to human protein C that was produced in our laboratory by the same techniques was used as a control in some experiments.

Effect of Mabs on HMWK Procoagulant Activity

Fifty microliters of serial dilutions of purified anti-HMWK Mabs (100, 50, 25, and 10 μg/mL), control Mabs (100 μg/mL) or TBS-BSA were incubated with equal volumes of NHP or purified HMWK (70 μg/mL) in 12 × 75-mm polystyrene tubes at 37 °C for one hour. The mixtures were then assayed for HMWK coagulant activity in comparison to a calibration curve made with serial dilutions of NHP, and the results were expressed as the percentage of activity relative to that found in the control.

Effect of Mabs on the Binding of HMWK to Kaolin

^{125}I-HMWK (10 μL) was incubated with 50 μL of NHP or purified HMWK (70 μg/mL), and 40 μL of Mabs (100 μg/mL) or

TBS-BSA, in a polystyrene tube for one hour at 37 °C. Then 100 μL of kaolin suspensions (10, 5, and 1 mg/mL, final concentrations) were added, and the mixtures were incubateed at 37 °C for 15 minutes. The tubes were centrifuged for two minutes at 5,000 g, and the kaolin pellet was counted for radioactivity. Binding of ^{125}I-HMWK was expressed as the percentage of the total radioactivity that was found in the pellet.

Immunoblotting

The original method of Towbin et al[24] was modified as follows: after a 7.5% SDS-PAGE in a discontinuous buffer system,[16] proteins were electrotransferred at 5 V/cm for two hours to a nitrocellulose membrane in a TE-52 Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco) filled with transfer buffer (50 mmol/L of Tris, 45 mmol/L of glycine, 20% vol/vol methanol, 0.02% SDS). After transfer, the nitrocellulose membrane was incubated in a blocking solution termed "BLOTTO"[25] consisting of 5% wt/vol nonfat dry milk, 0.01% antifoam A, 0.0001% merthiolate, 1 μmol/L of *p*-amidinophenyl-methyl-sulfonyl-fluoride, 0.02% NaN_3, in PBS. The same blocking solution was used in the following steps as incubation medium for the antibodies or as washing solution. Immunodetection of the antigens bound to the nitrocellulose membrane was then achieved by a double antibody reaction: the membrane was first reacted with Mabs (0.1 to 5 μg/mL) for three hours, washed three times (10 minutes each), and incubated for one hour with ^{125}I-labeled rabbit anti-mouse IgG antibodies. All the steps were performed at room temperature. The membrane was washed again three times with BLOTTO and once with PBS and was dried and exposed to x-ray films.

For the immunoblotting of HMWK in whole plasma, pooled NHP or individual plasma samples were diluted 1:5 in TBS-BSA; then 30-μL aliquots were mixed with an equal amount of sample buffer, boiled for five minutes, and applied to the gel.

For the development of a quantitative immunoblotting assay of HMWK in plasma, optimal conditions for the transfer of antigen were evaluated by measuring the recovery of ^{125}I-labeled HMWK on the nitrocellulose membrane. Increasing amounts of ^{125}I-HMWK, diluted in TBS-BSA or in plasma, were blotted onto nitrocellulose membranes at 5 V/cm, at transfer times of 1, 2, 3, and 4 hours. Sixty-five percent of the radioactivity applied to the gel could be recovered after a two-hour transfer, with a negligible increase after longer transfer times. In addition, the transfer was found to be proportional to the amount applied to the gel only when ^{125}I-HMWK added to NHP was diluted in HMWK-deficient plasma, but not when it was diluted in TBS-BSA, indicating that a constant amount of carrier plasma protein was essential to obtain a proportional transfer. Therefore, the reference curve for the quantitative assay was prepared by mixing 1:5 diluted NHP with 1:5 diluted HMWK-deficient plasma to obtain values of 80%, 60%, 40%, and 20% NHP in a constant amount of total plasma (6 μL). Individual plasma samples were similarly assayed by mixing equal volumes of the test plasma and HMWK-deficient plasma. After immunoblotting and exposure to x-ray films was done, the radioactivity corresponding to HMWK bands was counted in strips cut from the nitrocellulose membranes.

Contact Activation of Plasma for Immunoblotting Experiments

To evaluate whether the immunoblotting technique could detect the specific cleavage of HMWK in plasma, the contact system was activated in vitro, using glass or kaolin. In the glass activation experiments, 200 μL of plasma was incubated in a 12 × 75-mm glass tube for 15 minutes at 37 °C. Plasma was then diluted 1:5 in TBS-BSA, and 30-μL aliquots were mixed with equal amounts of sample buffer, boiled, and electrophoresed. In the kaolin activation experiments, 180 μL of plasma was incubated with 20 μL of a 200 mg/mL of kaolin suspension in polystyrene tubes at 37 °C for 15 minutes. After centrifugation at 5,000 g for two minutes, the supernatant was removed, and the kaolin pellet was washed twice with 200 μL of TBS-BSA containing 50 μg/mL of SBTI to quench the reaction. Proteins were extracted from the kaolin pellet by adding 200 μL of a 10% SDS solution. Six-microliter aliquots of kaolin supernatant and kaolin extract were then mixed with sample buffer, electrophoresed, and subjected to immunoblotting.

RESULTS

Purification of HMWK

The purified HMWK had a specific activity of 16 clotting units per milligram and migrated as a single band of 110,000 apparent mol wt when analyzed in a continuous buffer SDS-PAGE system, either in the presence or absence of reducing agents (Fig 1a). However, when the same preparation was electrophoresed in a discontinuous buffer SDS-PAGE system (Fig 1b), Coomassie blue stained two distinct protein bands, a major band with an apparent mol wt of 120,000 and a minor one with a mol wt of 105,000. Under reducing conditions, the major band showed a slightly faster mobility (~110,000 mol wt). When eluted from replicate SDS gels, both bands had HMWK clotting activity (~10 clotting units per milligram for the 120,000-mol-wt band, and ~2 clotting units per milligram for the 105,000-mol-wt band) and were cleaved by purified human kallikrein to give characteristic heavy and light chains on reduced SDS gels (data not shown).

After prolonged (60 minutes) incubation of the purified HMWK with purified plasma kallikrein, two closely migrating bands were generated with ~100,000 and 95,000 mol wt on nonreduced SDS gels (Fig 2). In the presence of a reducing agent, both bands disappeared to produce cleavage fragments having apparent mol wts of 62,000 and 50,000. These last two polypeptide chains, termed heavy and light chains, were separated by ion-exchange chromatography after reduction and alkylation of the cleaved molecule.[12] The 50,000-mol-wt fragment retained the original clotting activ-

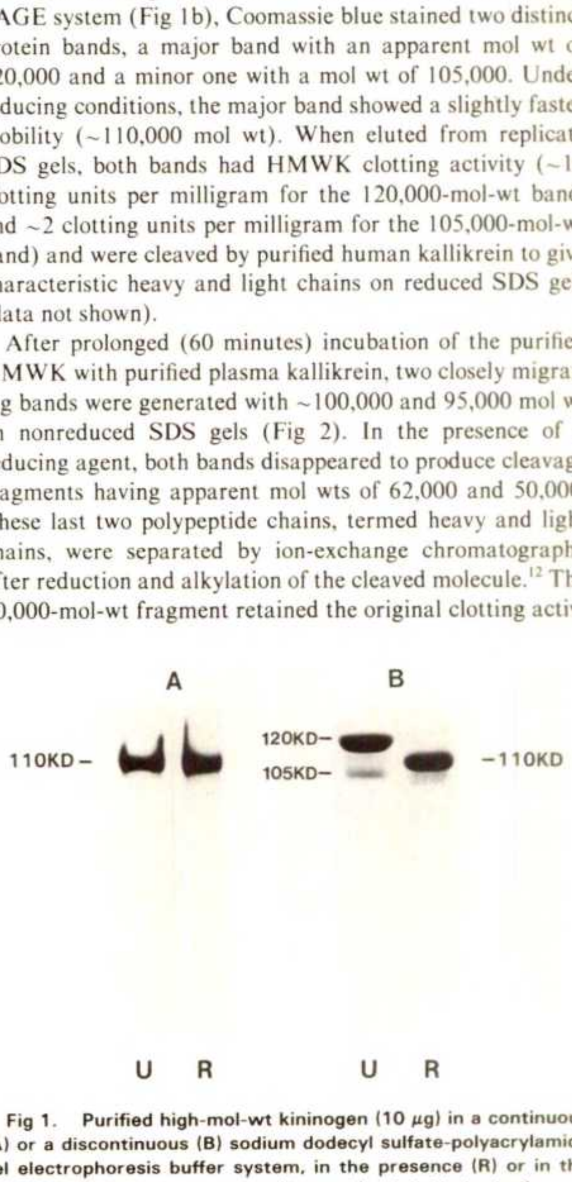

Fig 1. Purified high-mol-wt kininogen (10 μg) in a continuous (A) or a discontinuous (B) sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer system, in the presence (R) or in the absence (U) of a reducing agent (6 mmol/L of dithiothreitol).

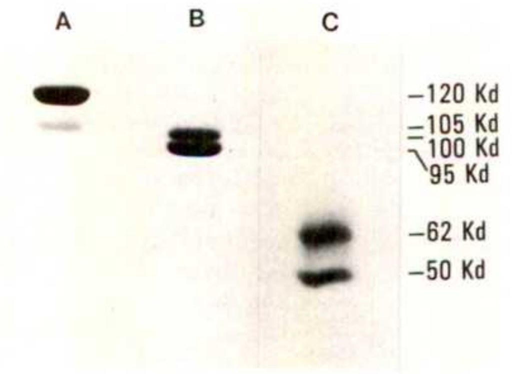

Fig 2. Cleavage of purified high-mol-wt kininogen (HMWK) by purified human plasma kallikrein. Lane (A) contains 8 μg of nonreduced HMWK incubated with Tris buffered saline (TBS). Eight micrograms of HMWK were incubated with kallikrein (80 ng) at 37 °C for one hour and then electrophoresed in a 7.5% discontinuous buffer sodium dodecyl sulfate-polyacrylamide gel electrophoresis system, in the absence (B) or in the presence (C) of 6 mmol/L of dithiothreitol.

ity of HMWK, whereas the 62,000-mol-wt fragment was inactive in a clotting assay. As evidenced by studies on the time course of HMWK cleavage by human plasma kallikrein, the 50,000-mol-wt fragment obtained after 60-minute incubation corresponds to the 45,000-mol-wt light chain described in previous reports.[13]

Mabs

Six Mabs (Table 1) were purified from ascitic fluids using protein A-Sepharose CL-4B affinity chromatography.[23] They appeared >95% homogeneous as judged by SDS-PAGE and were assigned to the different IgG subclasses by double immunodiffusion against specific rabbit antisera.

Studies were performed to determine which region of HMWK was recognized by the Mabs and whether they affected the functional activities of HMWK. By means of the immunoblotting technique, three of these Mabs were shown to recognize the heavy chain region of HMWK (Fig 3b), and one Mab recognized the alkylated light chain (Fig 3a). The remaining two Mabs did not bind to the light chain or the heavy chain regions, although both antibodies bound to both single-chain and two-chain unreduced HMWK. None of the purified Mabs affected either the clotting activity of HMWK or its binding to kaolin, either in a purified system or in plasma.

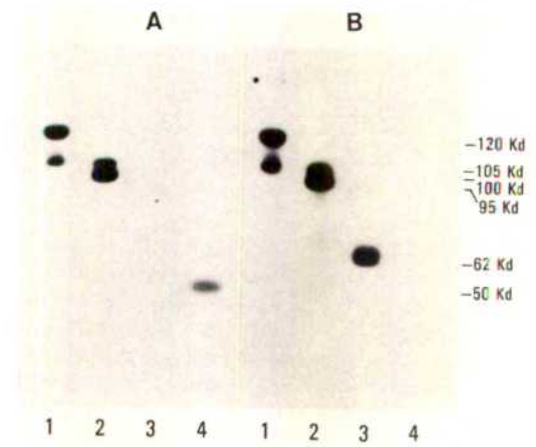

Fig 3. Immunoblotting of purified high-mol-wt kininogen (HMWK) and its cleavage derivatives using monoclonal antibodies (Mabs). One microgram of single-chain (1) or two-chain (2), kallikrein cleaved, HMWK, and 0.5 μg of the purified alkylated heavy chain (3) and light chain (4) were electrophoresed in a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis system and transferred to nitrocellulose membranes. For the immunodetection, each membrane was then reacted with different Mabs. The pattern shown on the left (A) was obtained with Mab H1D; the pattern on the right (B) was obtained with Mab H5A. Mabs H2D and H4D gave the same pattern as H5A.

Immunoblotting of HMWK in Plasma

Two Mabs, one (H5A) directed against the heavy chain region and one (H1D) directed against the light chain region of HMWK, were selected for further studies of the protein in plasma samples using the immunoblotting technique. Samples containing 6 μL of plasma were electrophoresed in a discontinuous buffer SDS-PAGE system, transferred to a nitrocellulose membrane, and then reacted with one of the two Mabs as described in the Materials and Methods section.

Mab H1D. Mab H1D detected principally two bands in normal human plasma (Fig 4) with apparent mol wts of ~120,000 and ~105,000, respectively, under nonreducing conditions (Fig 4, lane a). This same pattern was obtained with purified HMWK (Fig 3). Under reducing conditions, NHP (Fig 4, lane f) and purified single-chain HMWK showed two closely migrating bands of ~110,000 mol wt. An identical pattern was observed for normal plasma samples collected using different anticoagulants (sodium citrate or

Table 1. Characteristics of Six Anti-HMWK Monoclonal Antibodies

Characteristic	H1D	H2D	H3C	H4D	H5A	H6C
Protein A binding	+	+	+	+	+	+
Subclass	IgG_1-K	IgG_{2a}-K	IgG_{2a}-K	IgG_1-K	IgG_1-K	IgG_1-K
Binding to						
Single-chain HMWK	+	+	+	+	+	+
Double-chain HMWK	+	+	+	+	+	+
Isolated light chain	+	–	–	–	–	–
Isolated heavy chain	–	+	–	+	+	–
Inhibition of						
HMWK clotting activity	–	–	–	–	–	–
HMWK binding to kaolin	–	–	–	–	–	–

HMWK, high-mol wt kininogen.

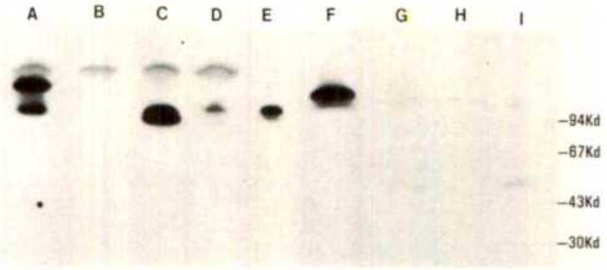

Fig 4. Immunoblotting of unactivated and activated human plasma using monoclonal antibody (Mab) H1D against the light chain region of high-mol-wt kininogen. Samples 6 μL were electrophoresed in a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis discontinuous system, transferred to a nitrocellulose membrane, and reacted with H1D Mab (5 μg/mL). The following samples were applied to the gel: normal human plasma (NHP), nonreduced (A) and reduced (F); nonreduced plasma from a patient with hereditary total kininogen deficiency (B); glass-activated NHP, nonreduced (C) and reduced (G); supernatant of kaolin-activated plasma, nonreduced (D) and reduced (H); kaolin pellet extract, nonreduced (E) and reduced (I). Glass and kaolin activation of plasma are discussed in the Materials and Methods section. Samples were reduced with 6 mmol/L of dithiothreitol.

heparin or EDTA) with or without the inhibitors (benzamidine, SBTI, leupeptin). The same pattern was observed in frozen pooled plasma or in plasma freshly collected from single individuals. Neither of the two bands was detectable in the plasma sample from an individual with hereditary total kininogen deficiency (Fig 4, lane b). A faint third band, with an apparent mol wt of 140,000 to 150,000, was sometimes detected in the unreduced plasma samples (Fig 4, lanes a through d), and it disappeared under reducing conditions (Fig 4, lanes f through h). For some normal plasma samples, this third faint band was not observed. Control experiments (data not shown) demonstrated that this band did not require the addition of the Mab and was not present in a plasma sample artificially depleted of IgG. Because this band comigrated with a band that was labeled by ^{125}I-protein A, it probably represented cross-reactivity of some batches of the ^{125}I-rabbit anti-mouse IgG with intact human IgG.

When normal human plasma was exposed to glass for 15 minutes at 37 °C to initiate contact activation before SDS-PAGE, an immunoblotting pattern similar to that of the purified two-chain, cleaved kininogen was observed (Fig 4, lane c), with two close bands that disappeared under reducing conditions (Fig 4, lane g), indicating that during contact activation of plasma HMWK was cleaved. A faint band of 50,000 mol wt corresponding to the light chain was detected by the H1D Mab under reducing conditions (Fig 4, lane g). The immunoblotting of the purified light chain of HMWK shown in Fig 3a, lane 4, was repeated under different experimental conditions. The signal detected in the autoradiographs was not modified when the same amount of light chain was pretreated with a reducing agent (6 mmol/L of DTT), but it was much weaker when the purified light chain was added to HMWK-deficient plasma, especially under reducing conditions. This experiment explains why the sensitivity of detection of the light chain of HMWK is decreased in reduced activated plasma samples.

Normal human plasma was also exposed to 20 mg/mL of kaolin, and the supernatant and kaolin-bound pellet were analyzed using immunoblotting techniques. As seen in Fig 4, most of HMWK originally present in NHP (lane a) was recovered from the kaolin pellet (Fig 4, lane e), with a small amount remaining in the kaolin supernatant (Fig 4, lane d). In both the kaolin extract and the kaolin supernatant, HMWK appeared as a two-chain molecule which, under reducing conditions (Fig 4, lanes h and i), dissociated to release the heavy chain (not detected by the monoclonal antibody used) and a light chain of 50,000 mol wt (Fig 4, lane i).

HMWK antigen in the plasma of a patient with a hereditary deficiency of C1 inhibitor was analyzed in plasma samples collected during and after an attack of angioedema (Fig 5). In the plasma sample collected from the patient on the first day of a severe attack of angioedema (Fig 5, lane b), HMWK was detected by the Mab H1D as a two-chain, cleaved molecule which was not detected under reducing conditions, implying that cleavage had occurred in vivo. Twenty-four hours later, when the patient was spontaneously recovering (Fig 5, lane c), and seven days following the initial day of the angioedema attack, when the patient was essentially asymptomatic (Fig 5, lane d), single-chain HMWK was detected, indicating the reappearance in plasma of the intact, uncleaved molecule.

Mab H5A. In addition to HMWK, the Mab H5A directed against the heavy chain region of HMWK recognized two additional bands in normal human plasma, either activated or not, but not in plasma deficient in both LMWK and HMWK (Fig 6). The two bands, with mol wts of ~60,000 and ~54,000, respectively, should therefore correspond to LMWK, whose heavy chain shares antigenic determinants with the heavy chain of HMWK.

Quantitative Immunoblotting Assay of HMWK in Human Plasma

Using the same two Mabs (H1D and H5A) in an immunoblotting technique, we developed a specific and sensitive

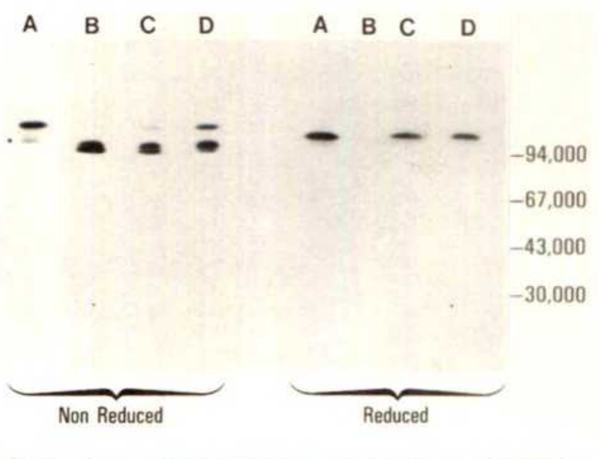

Fig 5. Immunoblotting of high-mol-wt kininogen (HMWK) in plasma from a patient with hereditary angioedema (C1-inhibitor deficiency), during (B) and (C), and seven days later near the end of an attack of angioneurotic edema (D). In lane (A), 6 μL of NHP were run for comparison. Samples on left were not reduced, whereas samples on right were reduced with 6 mmol/L of dithiothreitol.

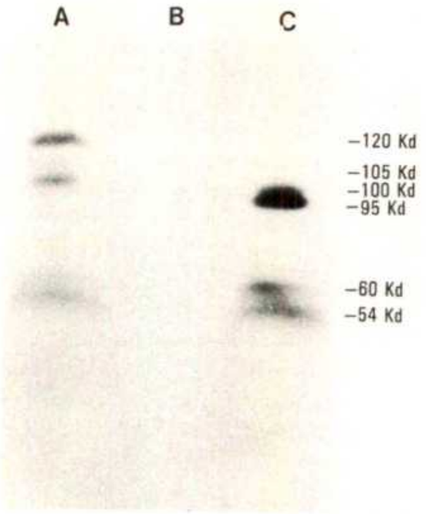

Fig 6. Immunoblotting of plasma using anti-heavy chain monoclonal antibody H5A (1 μg/mL). Each lane contained 6 μL of nonreduced plasma: (A) normal human plasma (NHP); (B) plasma from a patient with hereditary total kininogen deficiency; (C) glass-activated NHP.

assay of HMWK in plasma, as described in the Materials and Methods section. To assess the sensitivity of the method, decreasing amounts of purified HMWK were added to kininogen-deficient plasma, electrophoresed, transferred to a nitrocellulose membrane, and finally reacted with either of the two Mabs. After autoradiography was performed, the radioactivity bound to the nitrocellulose at the two bands of HMWK was counted. A linear relationship was found between the amount of HMWK added to the gel and the radioactivity counted on the nitrocellulose membrane, and as little as 10 ng of HMWK were clearly visible in the autoradiograph and gave counts three times higher than the control sample of HMWK deficient plasma (Fig 7).

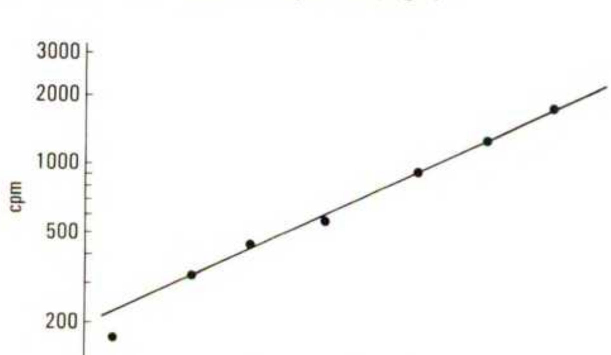

Fig 7. Quantitative immunoblotting of purified high-mol-wt kininogen (HMWK) in HMWK-deficient plasma. Varying amounts of HMWK were added to deficient plasma and subjected to immunoblotting analysis using monoclonal antibody H1D as described in the Materials and Methods section. The log–log plot shows the radioactivity (cpm) of ^{125}I-second antibody bound to the nitrocellulose membrane *v* amount of HMWK added to the polyacrylamide gel.

Similarly, in the immunoblotting of progressive dilutions of normal human plasma, the sensitivity of the technique was found to be ~3% of NHP (corresponding to 0.2 μL of plasma under our conditions). According to the immunoblotting technique, the concentration of HMWK in pooled normal plasma was ~55 μg/mL. Five replicate assays of plasmas from 17 male normal subjects gave a mean value of 100.7% ± 15.8% of pooled normal plasma with a mean interassay coefficient of variation of 15%. A good correlation ($r = .78$, $N = 17$, least-square regression) between the quantitative immunoblotting assay and the clotting assay of HMWK was also observed.

DISCUSSION

Since the first description of the immunoblotting of proteins,[24] various applications and technical improvements have been reported (for review, see ref 26). In immunoblotting studies of a complex protein mixture such as plasma,* the antibody probe used for the immunodetection of the antigen under investigation must be as specific as possible. Therefore, a series of murine hybridoma clones producing Mabs to human HMWK was prepared, and the purification and partial characterization of six Mabs is reported here. The specificity of these antibodies was first evaluated by a dot-immunobinding assay using the ascitic fluid and then by the immunoblotting of purified HMWK using the purified immunoglobulins. The specificity for kininogen of two Mabs (H1D and H5A) used here in the immunoblotting studies of plasma was confirmed by the observation that these antibodies did not recognize any protein in a plasma deficient in HMWK and LMWK. When the purified HMWK preparation used as immunogen to produce the murine monoclonal antibodies was analyzed in a discontinuous buffer SDS-PAGE system, two protein bands were detected, a major one with an apparent mol wt of 120,000 and a minor one with an apparent mol wt of 105,000. The question of whether the HMWK preparation contained a major contaminating protein that was not separated in the continuous buffer SDS-PAGE system was addressed by elution experiments. When eluted from unstained replicate gels, both the 120,000-mol-wt band and the 105,000-mol-wt band were able to shorten the prolonged clotting time of a HMWK deficient plasma, indicating that both contained HMWK clotting activity. In addition, the two bands were detected in normal plasma by both anti-light chain and anti-heavy chain HMWK Mabs, but not in LMWK- and HMWK-deficient plasma. Therefore, both the bands are HMWK. HMWK has been purified in several laboratories using different purification procedures and appeared as a homogeneous single-band protein when analyzed by a continuous phosphate-buffered SDS-PAGE system.[5,27] In some reports, however, the purified protein was electrophoresed in a discontinuous buffer SDS-PAGE system; under these conditions, two distinct bands were identified.[11,13] Mori and Nagasawa[13] considered the lower band as a cleavage derivative of HMWK produced during the purification procedure. This hypothesis may or may not be true, because our results show the presence of two bands of HMWK in plasma freshly collected in the presence of serine-protease or cysteine-protease inhibitors. Pierce and Guimaraes[28] and Thompson et al,[11] on the other hand, suggested that human HMWK may be heterogeneous, and our finding that HMWK is directly detectable as two bands in immunoblots of plasma is in accord with this hypothesis. Moreover, it is possible that a second form of single-chain HMWK can be identified that comigrates with cleaved unreduced HMWK.

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*Quantitative immunoblotting studies of plasma coagulation proteins have been described by our laboratory in preliminary reports at the Xth International Congress on Thrombosis and Hemostasis, San Diego, 1985, for HMWK, factor XII, protein S, protein C, and factor IX. Reported in abstract form in Thromb Haemost 54:338, 633, 710, 1354 (1985).

The in vitro cleavage of HMWK in plasma was studied using the immunoblotting technique with an anti-light chain Mab (H1D). In plasma subjected to contact activation by glass or kaolin, the unreduced cleaved HMWK was seen as two close bands near 100,000 apparent mol wt in a discontinuous SDS-PAGE system (Fig 4), and the immunoblotting of plasma essentially reproduced the pattern observed in SDS-gels of purified HMWK cleaved by purified human plasma kallikrein. Further evidence of HMWK cleavage was provided by the disappearance of the two bands in reduced plasma samples even if the cleavage fragment (the light chain of HMWK) was not always clearly detectable.

The ability of the immunoblotting technique to detect the in vitro cleavage of HMWK in plasma prompted us to evaluate whether the method was suitable for the detection of an in vivo cleavage of HMWK in pathologic conditions. In a preliminary study, we analyzed plasma samples from a patient with a hereditary deficiency of C1 inhibitor, a clinical condition characterized by recurrent attacks of mucocutaneous swelling and abdominal pain (hereditary angioneurotic edema).[29] The typical attacks have been generally attributed to the production of proinflammatory mediators derived from the complement cascade following the spontaneous and unimpeded activation of C1. However, a potential pathogenic role for kinin derived from the activation of the contact system has also been proposed.[30-32] Suction-induced blister fluids from patients with hereditary angioedema contain active plasma kallikrein.[31] In addition, peripheral blood collected during a typical angioedema attack contains decreased levels of plasma prekallikrein activity and of HMWK activity and antigen.[32] Further evidence is now provided by immunoblotting studies of plasma which show that during a typical attack of angioedema HMWK circulates in plasma as a two-chain, cleaved molecule (Fig 5).

The immunoblotting of plasma with a Mab directed against the heavy chain region of HMWK also detected LMWK. The immunoblots suggest that two different forms of LMWK exist in human plasma (Fig 6). The presence of two or more distinct forms of LMWK has been demonstrated in rat plasma[33] and has been suggested for human and bovine plasma.[28,34]

Immunoblotting studies presented here using Mabs allow the qualitative and quantitative investigation of HMWK in plasma. The results indicate that multiple molecular forms, both uncleaved and cleaved, can be identified in plasma without the need for purification or radiolabeling procedures. These immunoblotting techniques that monitor in vitro and in vivo cleavage of HMWK may be useful in obtaining new information on the structure and function of HMWK and its role in pathologic processes.

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Platelet Membrane Alterations Induced by the Local Anesthetic Dibucaine

By Ellinor I.B. Peerschke

Tertiary amine local anesthetics modify a variety of platelet membrane-related functions. The present study explored dibucaine (DB)-induced inhibition of platelet cohesion by examining structural and functional alterations of the human platelet membrane glycoprotein IIb-IIIa complex (GPIIb-IIIa) and platelet Ca^{2+} homeostasis. Complete inhibition of ADP-induced aggregation was achieved five minutes after platelet exposure to 0.10 to 0.25 mmol/L of DB when fibrinogen binding was reduced by 50%. At higher concentrations of DB (~1 mmol/L), ADP-induced fibrinogen binding was completely blocked. Scatchard analysis revealed loss of high-affinity binding sites in addition to reduction in B_{max}. In contrast, chymotrypsin-treated platelets sustained 50% inhibition of fibrinogen binding when incubated with 0.4 to 0.5 mmol/L DB, and kinetic analysis showed that the high-affinity platelet–fibrinogen interactions were reduced but not absent. Fibrinogen binding to chymotrypsin-treated platelets could not be completely inhibited even at high DB concentrations (1 mmol/L). The inhibition of fibrinogen binding to chymotrypsin-treated platelets correlated with changes in binding of a monoclonal antibody (10E5) specific for an epitope on the GPIIb-IIIa complex. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and radioelectroimmunoassay of DB-treated platelets, however, showed no evidence of a reduction or degradation of GP IIb or IIIa. Platelet incubation with DB (five minutes, 0.1 to 1.0 mmol/L) was also accompanied by: (*a*) increased platelet membrane-associated Ca^{2+} involving low-affinity binding sites [Kd = 5 × 10^{-5} mol/L-]; (*b*) increased $^{45}Ca^{2+}$ uptake which correlated with degradation of actin-binding protein (ABP) and digestion of GPIb as visualized on periodic-acid Schiff (PAS)-stained SDS gels and as inferred from decreased binding of a monoclonal antibody (6D1) directed against this glycoprotein; and (*c*) enhanced Ca^{2+} exchange. Thus, exposure of platelets to DB results in membrane-related alterations that may contribute to inhibition of platelet cohesion: (*a*) Decreased fibrinogen receptor exposure by traditional agonists and diminished accessibility of the GPIIb-IIIa complex to extracellular ligands correlate with DB-induced inhibition of platelet aggregation; and (*b*) increased calcium uptake and exchange across the platelet membrane likely leads to activation of the calcium-dependent protease(s) which was previously shown to correlate with DB-induced inhibition of ristocetin-induced platelet agglutination.

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IN ADDITION to their well-known inhibitory effects on neuronal responses, tertiary amine local anesthetics have been reported to modify a variety of non-neuronal activities including ovum activation,[1,2] exocytosis,[3] endocytosis,[4] anion transport in erythrocytes,[5,6] cell spreading and motility,[7,8] von Willebrand factor (VWF)-mediated platelet agglutination,[9] fibrinogen-mediated platelet aggregation, and the platelet release reaction.[10] They have also been shown to dissolve platelet cytoskeletal components,[11] inhibit protein kinase C[12] and phospholipase A_2,[13] interfere with platelet adhesion to fibrinogen-coated beads[14] and, depending on concentration, to potentiate or inhibit ristocetin-induced platelet agglutination.[9] The precise mechanism of action of local anesthetics, however, remains unclear. Some of their effects on cellular activity and reactivity have been attributed to the ability of local anesthetics to perturb membrane phospholipid organization, bilayer fluidity, and membrane protein conformation.[15] Other effects have been ascribed to their ability to displace calcium from various cell membranes and interfere with cellular calcium metabolism.[16] In fact, it has been suggested[17] that local anesthetics affect platelet aggregation by modifying the participation of calcium in this process. The present study was thus designed to investigate further the mechanism of local anesthetic inhibition of platelet cohesion by evaluating the effects of dibucaine (DB) on the structural and functional integrity of the platelet membrane glycoprotein IIb–IIIa (GPIIb–IIIa) complex, on calcium homeostasis, and on the relationship between platelet calcium homeostasis and degradation of GPIb.

MATERIALS AND METHODS

Platelet preparation. Blood was obtained according to the guidelines of the Declaration of Helsinki from healthy volunteers who had not taken aspirin for at least eight days. It was anticoagulated with 0.1 vol 3.2% sodium citrate. Platelet-rich plasma (PRP) was prepared by low-speed centrifugation (280 g, 15 minutes). Gel-filtered platelets (GFP) suspended in HEPES-buffered modified Tyrode's solution, pH 7.5 (HBMT) were prepared from PRP as described previously.[18] GFP were incubated with either 0.15 mol/L of saline or 0.1 to 1.0 mmol/L of DB (Sigma Chemical Co, St Louis, lot No. D-0638) for five to 60 minutes at 22 °C.

Structure and function of GPIIb–IIIa. The integrity of the GPIIb–IIIa complex was assessed by: (a) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% slab gels)[19] and autoradiography of ^{125}I-labeled platelets,[20] (b) binding of a monoclonal antibody (10E5) that recognizes an epitope on the calcium-dependent GPIIb–IIIa complex,[21] and (c) radioelectroimmunoassay of the GPIIb–IIIa antigen of solubilized platelets as detected by radiolabeled 10E5. ^{125}I-Labeled platelets[20] treated with DB or 0.15 mol/L of NaCl were prepared for SDS-PAGE by solubilizing in a buffer containing one part 3.3% SDS, 6 mmol/L of *N*-ethylmaleimide, and one part 1% SDS, 12.5 mmol/L Tris/Cl, 20% glycerol, and 0.025% bromophenol blue, pH 6.8.

Platelets for radioelectroimmunoassays were prepared from GFP in HBMT following incubation with 0.15 mol/L of NaCl or DB by being washed three times with 0.15 mol/L of NaCl, 0.01 mol/L of Tris/Cl, 1 mmol/L of EDTA, and 5 mmol/L of glucose, pH 7.4. They were resuspended to a count of 6 × 10^{12} platelets/L in 0.1 mol/L of glycine, 0.038 mol/L of Tris, pH 8.7, solubilized by the addition of 0.1 vol of 10% Triton X-100 (Eastman Kodak, Roches-

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ter, NY) and by being rocked at 4 °C for 30 minutes, and centrifuged at 22 °C for five minutes at 12,000 g in a Beckman microfuge (Beckman Instruments Corp, Palo Alto, Calif) to remove remaining debris. Assays were performed in 1% agarose gels containing a combination of 1% Triton X-100, IgG prepared from the serum of rabbits immunized with whole platelets (Accurate Chemicals, Westbury, NY, Lot 073A) and radiolabeled 10E5 antibody (6×10^5 cpm; specific activity 98 cpm/ng). Samples (5 μL) were electrophoresed at 8 mA for 30 minutes and then at 19.5 mA for 18 hours. The gels were sequentially soaked in 0.15 mol/L of NaCl and water, dried on a support medium (Gelbond, FMC Corp, Rockland, Mass) with the aid of an electric drier, stained with 0.5% Coomassie blue in 45% methanol–10% acetic acid, destained for three to five minutes in the same solution without Coomassie blue, redried, and subjected to radioautography for various lengths of time at −80 °C using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) and an intensifying screen (Lightning-Plus, Dupont, Wilmington Del).

The functional integrity of the GPIIb–IIIa complex was assessed by: (a) comparing the ability of control and DB-treated platelets to bind fibrinogen following stimulation with 10 μmol/L of ADP[18] or treatment with chymotrypsin (Sigma) (500 μg/mL, 15 minutes, 22 °C),[22] and (b) evaluating fibrinogen-mediated aggregation of control and DB-treated platelets using a Chronolog (Haverton, Pa) dual-channel aggregometer whose baselines were set at 10% light transmission using the unstimulated GFP suspension and 90% light transmission using a buffer blank. All functional studies were performed at room temperature.

LDH release and PF3 availability. Release of lactate dehydrogenase (LDH) from the platelet cytoplasm was measured with a Dupont ACA (Clinical Systems Division, Dupont, Wilmington, Del). Platelet factor 3 (PF3) availability was measured by preincubating a mixture of pooled normal plasma at a 1:1 ratio (George King Biomedical Co, Overland Park, Kan) and kaolin (Sigma) for five minutes at 37 °C and then adding 0.2 mL of the mixture to 0.2 mL of GFP (150,000/μL). $CaCl_2$ (0.1 mL of a 0.035 mol/L stock solution) was added 30 seconds later, and clot formation was monitored using a semiautomated impedance device (fibrometer, BBL, Division of Becton Dickinson Co, Cockeysville, Md).

Platelet calcium homeostasis. DB-induced changes in calcium homeostasis were assessed and correlated with degradation of platelet membrane GPIb and actin-binding protein (ABP). The effect of DB in calcium homeostasis was evaluated by characterizing: (a) $^{45}Ca^{2+}$ (Amersham Corp, Arlington Heights, Ill) binding and uptake of platelets in calcium-free and magnesium-free HBMT[23] to which increasing concentrations of ^{45}Ca were added, and (b) Ca^{2+} exchange under steady-state conditons.[24] Scatchard plots of Ca^{2+} binding were constructed with the aid of a Hewlett Packard HP-85 curve-fitting program (Hewlett Packard, Corvallis, Ore) and analyzed according to the methods of Rosenthal[25] and Feldman.[26] Platelet membrane-associated calcium was defined as platelet-associated calcium that was removed following five minutes of incubation with 10 mmol/L of EDTA.[23] Ca^{2+} exchange was quantified by measuring Ca^{2+} influx and efflux as follows: GFP in Ca^{2+}-free and Mg^{2+}-free HBMT were incubated for 30 minutes at 22 °C with 0.2 mmol/L of unlabeled Ca^{2+} and subsequently loaded with tracer amounts of $^{45}Ca^{2+}$ (17.55 mCi/mg; 0.5 to 1.0×10^7 cpm/mL in the presence and absence of DB. Ca^{2+} uptake was assessed at timed intervals up to 120 minutes by centrifugation of platelets through silicone oil. Duplicate samples were subjected to an additional five-minute incubation with 10 mmol/L of EDTA before centrifugation. Following Ca^{2+} uptake, platelet preparations were acidified to pH 6.5 using 0.1 N HCl, centrifuged (1,000 g, 15 minutes, 22 °C), and resuspended in buffer containing 1.0 mmol/L of unlabeled Ca^{2+}. Ca^{2+} efflux was subsequently monitored at time intervals up to 120 minutes as above, both before and after five-minute incubation with 10 mmol/L of EDTA.

Because the GPIIb–IIIa complex has been implicated in calcium homeostasis,[23,27] additional studies were performed in which platelets were pretreated with either 10 mmol/L of EDTA or the combination of 10 mmol/L of EDTA and 10 mmol/L of unlabeled $CaCl_2$ at 37 °C and ph 8.1 for 60 minutes. The ability of EDTA-treated platelets with dysfunctional GPIIb–IIIa complexes to take up ^{45}Ca in response to exposure to 1.0 mmol/L of DB was monitored after five and 30 minutes and compared with that of control platelets.

Degradation of ABP and platelet membrane GP Ib was assessed following platelet incubation with DB in the presence and absence of 10 mmol/L of EDTA. Platelets were lysed in a solution containing one part 3.3% SDS, 6 mmol/L of *N*-ethylmaleimide, and one part 1% SDS, 12.5 mmol/L Tris (hydroxymethyl) aminomethane chloride, 20% glycerol, and 0.025% bromophenol blue (pH 6.8), heated to 100 °C for three minutes, and electrophoresed into 7.5% SDS-polyacrylamide gels.[19] The gels were stained for protein with Coomassie blue or for carbohydrate with PAS.[28] PAS-stained gels were scanned using a Beckman Model CDS 200 gel scanner. DB-induced alterations of platelet membrane GPIb were also monitored by comparing the ability of a radiolabeled monoclonal antibody (6D1) specific for this glycoprotein[29] to bind to control and DB-treated platelets.

RESULTS

Structural and functional integrity of the platelet membrane GPIIb–IIIa complex. DB reduced the binding of a monoclonal antibody (10E5), specific for an epitope on the GPIIb–IIIa complex in a concentration-dependent manner within five minutes of platelet exposure to the anesthetic (Fig 1A). Maximum inhibition (~50%) occurred at DB concentrations >0.5 mmol/L. The antibody recognized 58,174 ± 14,744 sites on control platelets, but only 23,247 ± 9877 sites (mean ± SD, N = 16) on platelets treated with 0.5 mmol/L of DB. This inhibition was not reversed when DB was removed by platelet centrifugation and resuspension in fresh buffer: 54% ± 5% (mean ± SD, N = 4) at five minutes and 52% ± 3% at 30 minutes. Platelet incubation with DB for up to 30 minutes, however, had no effect on platelet membrane GPIIb or GPIIIa as assessed by SDS-PAGE (data not shown). Moreover, when platelets were lysed with Triton X-100 and electrophoresed into 1% agarose gels containing a mixture of unlabeled rabbit anti-whole platelet antiserum and ^{125}I-labeled 10E5, the 10E5 antibody recognized similar quantities of GPIIb–IIIa complexes in control and DB-treated platelet samples (Fig 2). Similar results were obtained when lysed platelets were incubated with the local anesthetic. Furthermore, when 10E5 was bound to platelets before exposure to DB, it did not dissociate on addition of the anesthetic (Table 1).

The effects of DB on platelet aggregation, fibrinogen binding, and 10E5 binding are correlated in Fig 1A. Preincubation (five minutes, 22 °C) of platelets with 0.1 to 0.25 mmol/L of DB resulted in complete inhibition of ADP-induced aggregation but in only 50% inhibition of fibrinogen binding. ADP-induced fibrinogen binding was almost completely inhibited at DB concentrations >0.5 mmol/L, as was fibrinogen binding induced by epinephrine, thrombin, and the combination of ADP and epinephrine (Table 2). ADP-

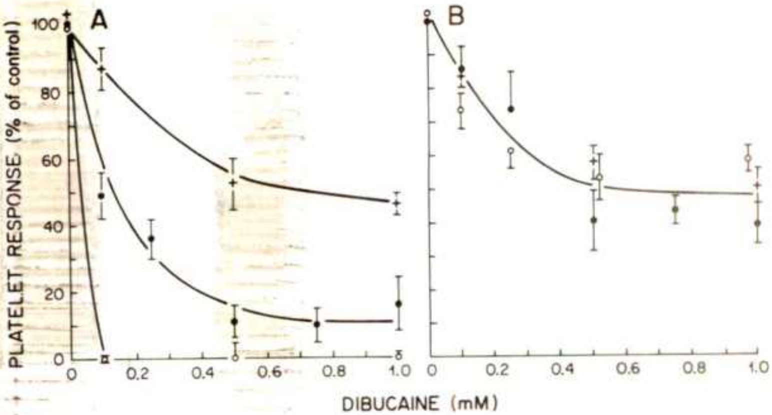

Fig 1. Comparison of the effect of dibucaine (DB) on 10 μmol/L ADP (A) and chymotrypsin (500 μg/mL for 15 minutes at 22 °C); (B) treated platelets. Values for platelet aggregation (extent) in the presence of 0.1 mg/mL fibrinogen (○), ^{125}I-fibrinogen binding at saturation (1.0 mg/mL) (●), and ^{125}I-10E5 binding near saturation (10 μg/mL) (+) are presented relative to control platelet responses. Platelets were incubated with increasing DB concentrations for five minutes at 22 °C, then stimulated with ADP or chymotrypsin. Data points represent the mean ± SD of 16 separate determinations.

induced fibrinogen binding was similarly inhibited if platelets were first stimulated with 10 μmol/L ADP and then exposed to the anesthetic (Table 3).

In contrast, platelets treated with chymotrypsin were minimally affected by low concentrations of DB (0.1 mmol/L) (Fig 1B). Chymotrypsin-induced platelet aggregation and fibrinogen binding were reduced in parallel following platelet exposure to higher DB concentrations. Neither decreased below ~50% of control levels, however, even at DB concentrations exceeding 0.5 mmol/L.

Scatchard analysis of fibrinogen binding to ADP-stimulated platelets indicated that DB not only decreased total receptor availability, but also specifically obliterated high-affinity fibrinogen binding (Fig 3A). In the presence of DB fibrinogen binding to ADP-treated platelets consistently yielded straight-line Scatchard plots as compared with curvilinear plots obtained with platelets in the absence of DB (Fig 3A), or platelets exposed to DB and stimulated with chymotrypsin (Fig 3B). Platelets stimulated with ADP bound 23,400 ± 6,852 (mean ± SD, N = 5) molecules of fibrinogen [Kd = 1.04 ± 0.6 μmol/L] in the presence of 0.1 mmol/L of DB, as compared with 49,300 ± 10,623 molecules of fibrinogen bound to control platelets in an apparently biphasic manner[30] (initial dissociation constant = 0.10 ± 0.05 μmol/L). Chymotrypsin-treated platelets bound 35,418 ± 11,072 molecules of fibrinogen with an initial high-affinity dissociation constant of 0.09 ± 0.05 μmol/L in the presence of 0.4 mmol/L of DB, and 66,397 ± 12,464 molecules of fibrinogen with an initial high-affinity dissociation constant of 0.08 ± 0.06 μmol/L in the absence of anesthetic.

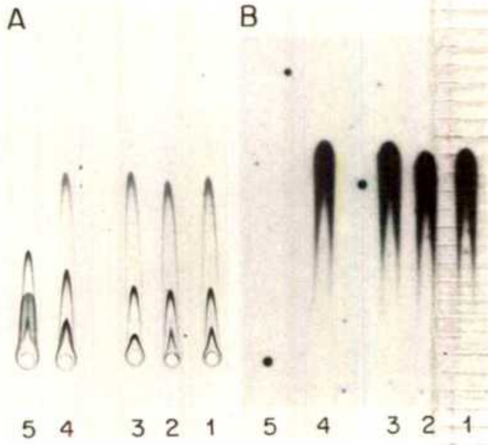

Fig 2. Radioelectroimmunoassay of solubilized platelets pretreated (five minutes at 22 °C) with either isotonic saline (lane 1), 0.1 mmol/L of dibucaine (DB) (lane 2), 0.5 mmol/L of DB (lane 3), or 1.0 mmol/L of DB (lane 4). Control platelets were also lysed and electrophoresed in the presence of 10 mmol/L of EDTA (lane 5). All samples were electrophoresed into a 1% agarose gel containing 0.29 mg/mL of an IgG fraction of rabbit anti-platelet serum and ^{125}I-labeled 10E5 antibody. (A) Coomassie blue-stained gel. (B) Corresponding autoradiogram.

LDH release and PF3 availability. The effect of DB on PF3 activity and lactate dehydrogenase (LDH) release are shown in Table 4. Increases in PF3 activity were already noted five minutes after platelet exposure to even the lowest DB concentrations (0.1 to 0.25 mmol/L), when virtually no LDH release was detectable, and continued to increase at higher DB concentrations. LDH release required prolonged platelet exposure (30 to 60 minutes) to DB concentrations exceeding 0.5 mmol/L.

Platelet calcium homeostasis. Because platelet incubation with DB has been shown to lead to degradation of both ABP and GPIb by activation of an enzyme(s) that is at least partially sensitive to calcium concentrations,[9,31] experiments were performed to investigate DB-induced alterations of platelet Ca^{2+} homeostasis. Although 6D1 binding to platelets was decreased in a concentration-dependent manner five minutes after platelet exposure to DB and continued to decrease during a subsequent 30-minute exposure to the

Table 1. Effect of 1 mmol/L of DB on the Dissociation of Platelet-Bound ^{125}I-10E5

Time* (min)	10E5 Binding† (% Relative to T_0)
0	100
2	98 ± 4
5	102 ± 2
15	101 ± 5
30	99 ± 3

*Platelets were incubated with 10 μg/mL ^{125}I-10E5. After 15 minutes 10E5 binding was assessed (T_0) and dibucaine (DB) was added. 10E5 binding was quantified at the designated times using aliquots of the platelet suspension.

†Mean ± SD, N = 3

Table 2. Effect of 0.5 mmol/L of DB on Fibrinogen Receptor Exposure by Various Agonists

Agonist	Fibrinogen Binding (% of Control)*
ADP (10 μmol/L)	18 ± 3
Epinephrine (10 μmol/L)	5 ± 7
Thrombin† (100 mU/mL)	21 ± 10
ADP (10 μmol/L) and epinephrine (10 μmol/L)	14 ± 4
Chymotrypsin (500 μg/mL, 15 minutes, 22 °C)	49 ± 15

*Mean ± SD, N = 5.

†Human thrombin was the gift of Dr John Fenton, New York State Department of Health, Albany.

All platelets were incubated with dibucaine (DB) for 5 minutes before stimulation.

Table 3. ADP-Induced Fibrinogen Binding

Platelets	Fibrinogen Binding (% of Control)*
Incubated with DB before stimulation	46 ± 16
Incubated with DB after stimulation	54 ± 15

Effect of 0.1 mmol/L of dibucaine on fibrinogen receptor exposure and fibrinogen binding to previously exposed receptors. Platelets were stimulated with 10 μmol/L of ADP; fibrinogen binding was assessed in the presence of 1.0 mg/mL ^{125}I-fibrinogen.

*Mean ± SD, $N = 3$.

anesthetic, measurable $^{45}Ca^{2+}$ uptake required either five-minute exposure of platelets to DB concentrations >0.5 mmol/L or 30-minute exposure of platelets to DB concentrations exceeding 0.1 mmol/L (Fig 4). DB-induced Ca^{2+} uptake correlated well with degradation of ABP (Fig 5). In contrast, PAS-stained polyacrylamide gels showed that GPIb degradation required at least a 60-minute exposure of platelets to DB at 0.5 mmol/L or 30-minute exposure to DB concentrations >0.5 mmol/L. Under these conditions, LDH release was also present (Table 4). As reported by others,[9,31] DB-induced degradation of both ABP and GPIb could be inhibited in the presence of EDTA (10 mmol/L).

The effect of 1 mmol/L of DB on platelet Ca^{2+} influx and efflux under steady-state conditions is shown in Fig 6A and B. Both the rate of Ca^{2+} influx and efflux increased markedly in the presence of 1 mmol/L of DB. Control platelets (5×10^7) took up Ca^{2+} at a rate of 0.65 ± 0.20 pmol/min, whereas DB-treated platelets (5×10^7) took up Ca^{2+} at an initial rate of 4 ± 1.2 pmol/min. Similar initial Ca^{2+} efflux rates were calculated, 0.55 ± 0.18 pmol/5×10^7platelets/min for control platelets, and 4.5 ± 1.6 pmol/5×10^7 platelets per minute for DB-treated platelets, indicating that $^{45}Ca^{2+}$ measurements were indeed made under equilibrium conditions.

Most DB-induced calcium uptake was mediated by the platelet membrane GPIIb–IIIa complex, as illustrated in Fig 6C. Platelets that were incubated with EDTA for 60 minutes at 37 °C and an alkaline pH to disrupt GPIIb–IIIa complexes[32] were previously reported to take up less Ca^{2+} than control platelets, but amounts similar to those taken up by platelets from patients with thrombasthenia.[23] In this study, EDTA-treated platelets also took up significantly less calcium than did control platelets following exposure to DB.

In addition to calcium uptake, exposure of platelets to DB also altered the fraction of platelet associated $^{45}Ca^{2+}$ that could be rapidly removed by EDTA and was thus presumed to be located on the surface membrane. Figure 7 depicts the binding of $^{45}Ca^{2+}$ to platelets before and after five minutes of exposure to 1 mmol/L of DB. Calcium binding was quantified two minutes after addition of increasing concentrations of $^{45}Ca^{2+}$, since previous studies indicated that most of the $^{45}Ca^{2+}$ associated with platelets under these conditions was surface bound.[33] As reported earlier,[33] platelets depleted of loosely associated surface Ca^{2+} following brief Ca^{2+} deprivation rebound $^{45}Ca^{2+}$ in an apparently biphasic manner, suggesting the presence of at least two membrane-associated Ca^{2+}-binding compartments. The first approached saturation near 100 μmol/L of free Ca^{2+}, whereas the second failed to saturate even at 1 mmol/L of free Ca^{2+}. Present data indicate that Ca^{2+} binding to both compartments appears to increase following platelet exposure to DB. Approximately $5 \times 10^5 \pm 1.57 \times 10^5$ (mean ± SD, N = 3) molecules of Ca^{2+} bound to the saturable compartment of control platelets with

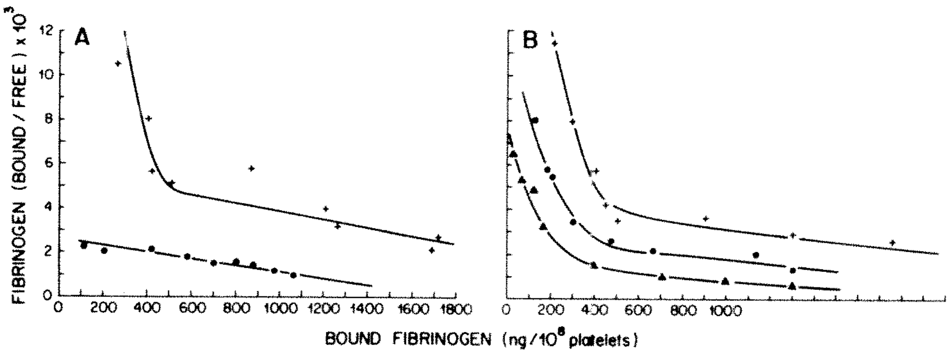

Fig 3. Scatchard analysis of fibrinogen binding to control platelets (x) and platelets incubated (five minutes at 22 °C) with 0.1 mmol of dibucaine (DB) (●) resulting in half-maximal inhibition of ADP-induced fibrinogen binding, or 0.4 mmol/L of DB (▲) causing half-maximal inhibition of chymotrypsin-induced fibrinogen binding. (A) Platelets stimulated with ADP (10 μmol/L); (B) platelets stimulated with chymotrypsin (500 μg/mL for 15 minutes at 22 °C). Results depict one of five similar experiments.

Table 4. Effect of Increasing DB Concentrations on Platelet PF3 Availability, LDH Release, and GPIb Degradation

	PF3 Availability (Kaolin Recalcification Time [sec]*)	LDH Release (% of Total)*			Glycoprotein Ib Integrity§ (% of Control)*		
					(+) EDTA	(−) EDTA	
DB (mmol/L)	5 Min†	5 Min†	30 Min†	60 Min†	60 Min†	30 min†	60 min†
0	107 ± 5‡	0	0	0	100	100	100
0.10	100 ± 5‡	0	2 ± 1	10 ± 3	—	—	—
0.25	90 ± 6	0	5 ± 2	15 ± 4	—	—	—
0.50	73 ± 4	0 ± 1	8 ± 2	30 ± 7	93 ± 3	94 ± 10	66 ± 14
0.75	68 ± 5	3 ± 1	30 ± 6	50 ± 8	83 ± 9	64 ± 12	29 ± 18
1.00	65 ± 3	5 ± 2	42 ± 5	56 ± 7	—	—	—

Abbreviations: DB, dibucaine; LDH, lactate dehydrogenase.

*Mean ± SD, n = 5.

†Incubation time with DB (22 °C).

‡Statistically significant difference using paired Student's *t* test, $p < 0.02$.

§Washed platelets were incubated with isotonic saline, 0.5 mmol/L, and 0.75 mmol/L DB in the presence and absence of 10 mmol/L EDTA. They were subsequently pelleted, solubilized in sodium dodecyl sulfate (SDS), electrophoresed into 7.5% SDS-polyacrylamide gels, stained with periodic acid Schiff reagent, and scanned at 525 nm. GPIb integrity of DB-treated platelets is expressed compared to that of control platelets incubated with saline on the basis of peak heights derived from densitometer scans.

an initial high-affinity dissociation constant of $2.0 \pm 1.0 \times 10^{-5}$ mol/L, as compared with $8 \pm 3 \times 10^{6}$ sites occupied by $^{45}Ca^{2+}$ on DB-treated platelets with an initial apparent high-affinity dissociation constant of $5 \pm 2.5 \times 10^{-5}$ mol/L. The difference in high-affinity dissociation constants was statistically significant using the paired Student's *t* test at $P < .05$.

DISCUSSION

The mechanisms by which tertiary amine local anesthetics affect various platelet membrane-related functions, including platelet adhesion to foreign surfaces,[16,34] platelet agglutination,[9] platelet aggregation, and the release of platelet granule contents,[10] have been of interest because understanding of these mechanisms is likely to enhance present knowledge of the biochemical and biophysical events involved in normal platelet cohesion. Previous studies with other cell systems have shown that the ability of anesthetics to interact with membrane lipids[11] and to displace membrane-bound calcium correlates with anesthetic potency,[12] and that the anesthetics may interact directly with membrane proteins[35,36] to inhibit their function.

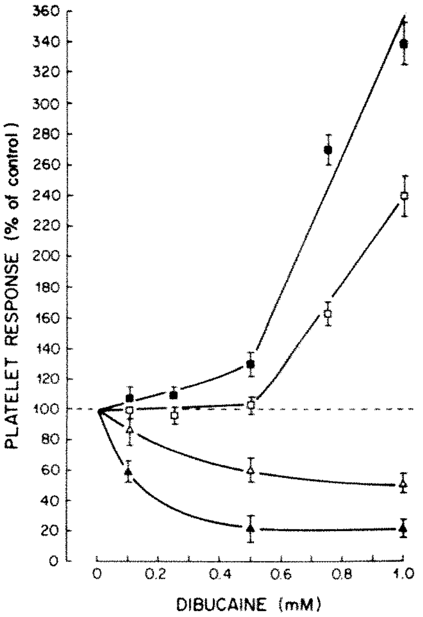

Fig 4. Comparison of ^{45}Ca uptake (squares) and ^{125}I-6D1 binding (triangles) (5 µg/mL, final concentration) by platelets incubated with increasing dibucaine (DB) concentrations for five minutes (open symbols) or 30 minutes (closed symbols). Data represent the mean ± SD of nine separate experiments.

Because local anesthetics modify platelet functions mediated both by the membrane GPIIb–IIIa complex and GPIb, and it has been suggested that the inhibition of platelet function by these agents results from changes in platelet membrane-associated and/or cytosolic calcium,[9,17] the present study examined platelet membrane alterations accompanying the DB-induced loss of platelet cohesion with emphasis on anesthetic-induced structural and functional alterations of the GPIIb–IIIa complex, and on platelet calcium homeostasis. The data have implications for the cellular control of platelet membrane-associated structures involved in fibrinogen-dependent platelet aggregation, provide direct evidence for DB-induced changes in Ca^{2+} homeostasis, and thus a mechanism whereby the platelet calcium-dependent protease(s) is activated to digest GPIb.

Brief incubation (five minutes) of platelets with low doses of DB (0.1 mmol/L) resulted in complete inhibition of ADP-induced platelet aggregation and a 50% reduction in fibrinogen binding. This reduction in fibrinogen binding was accompanied not only by decreased fibrinogen receptor availability, but also by the absence of high-affinity fibrinogen binding. Recent studies using monoclonal antibodies have shown that both high-affinity and low-affinity platelet fibrinogen interactions are likely mediated by the GPIIb–IIIa complex[20] and suggest that differences in affinity may reflect (a) heterogeneity of the microenvironment surrounding the GPIIb–IIIa complexes; (b) the presence of at least two different platelet-binding domains on fibrinogen molecules; and/or (c) interactions among fibrinogen receptors

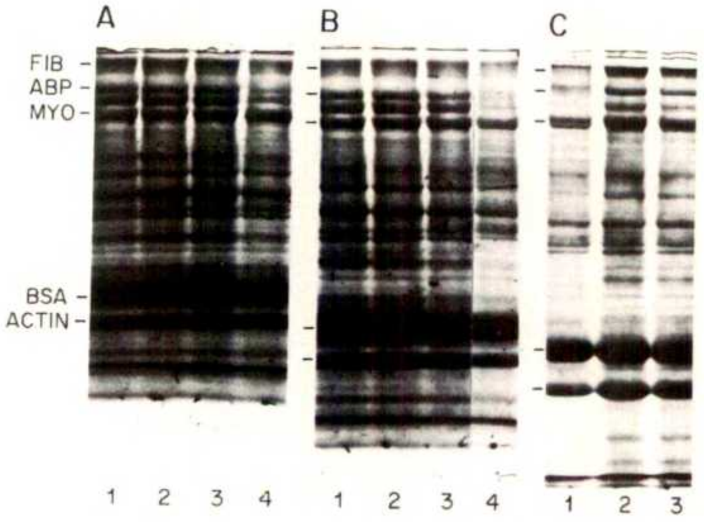

Fig 5. Nonreduced 7.5% sodium dodecyl sulfate polyacrylamide gel of platelets treated with dibucaine (DB) in the presence and absence of EDTA illustrating the degradation of actin-binding protein. (A) Platelets incubated for five minutes with isotonic saline (lane 1), 0.1 mmol/L of DB (lane 2), 0.5 mmol/L of DB (lane 3), and 1.0 mmol/L of DB (lane 4). (B) Platelets incubated for 30 minutes with isotonic saline (lane 1), 0.1 mmol/L of DB (lane 2), 0.5 mmol/L of DB (lane 3), and 1.0 mmol/L of DB (lane 4). (C) Platelets incubated for 30 minutes with 1.0 mmol/L of DB (lane 1), isotonic saline (lane 2), and 1.0 mmol/L of DB (lane 3) in the presence of 10 mmol/L of EDTA. Gels were stained with Coomassie Brilliant Blue and photographed.

resembling negative cooperativity.[37] Because high-affinity platelet–fibrinogen interactions appear to be essential for platelet aggregation, and reach a maximum at fibrinogen concentrations supporting maximal platelet aggregation (100 to 200 μg/mL),[18] their loss may explain the apparent discrepancy between ADP-induced fibrinogen binding and platelet aggregation observed in the present study. This interpretation is supported by the finding that chymotrypsin-treated platelets incubated with DB also suffered a reduction in total fibrinogen binding, but high-affinity platelet fibrinogen interactions remained intact, albeit reduced, as did platelet aggregation. It is postulated that the DB-induced effect on high-affinity fibrinogen binding to platelets stimulated with ADP results from anesthetic-induced alterations of the GPIIb–IIIa microenvironment by direct or indirect modification of platelet membrane constituents that are degraded during fibrinogen receptor exposure by chymotrypsin.

Differences in the extent to which local anesthetics inhibit fibrinogen binding of ADP-treated and chymotrypsin-treated platelets suggest furthermore that certain events contributing to ADP-induced activation transduction may be additionally sensitive to local anesthetics. The mechanism of fibrinogen receptor exposure by chymotrypsin is different from ADP-induced fibrinogen receptor exposure.[38] This hypothesis is supported by data from a recent study demonstrating that DB inhibits ADP-induced increases in binding of a monoclonal antibody (7E3) whose rate of binding to platelet GPIIb–IIIa complexes is activation dependent.[39] Comparison of the effect of DB on fibrinogen and 10E5 binding to platelets stimulated with ADP and chymotrypsin thus points toward alteration of both GPIIb–IIIa receptor exposure mechanisms (ADP-treated platelets) and receptor accessibility to ligand (chymotrypsin-treated platelets).

In the present study, differences between the DB-induced inhibition of ADP and chymotrypsin-induced fibrinogen binding mimicked those reported when platelets were treated with cytochalasins.[40,41] ADP-induced exposure of fibrinogen receptors and maintenance of the receptors in an exposed state were inhibited by cytochalasins, suggesting that they were dependent on platelet cytoskeletal elements.[40,41] In contrast, chymotrypsin-treated platelets did not appear to

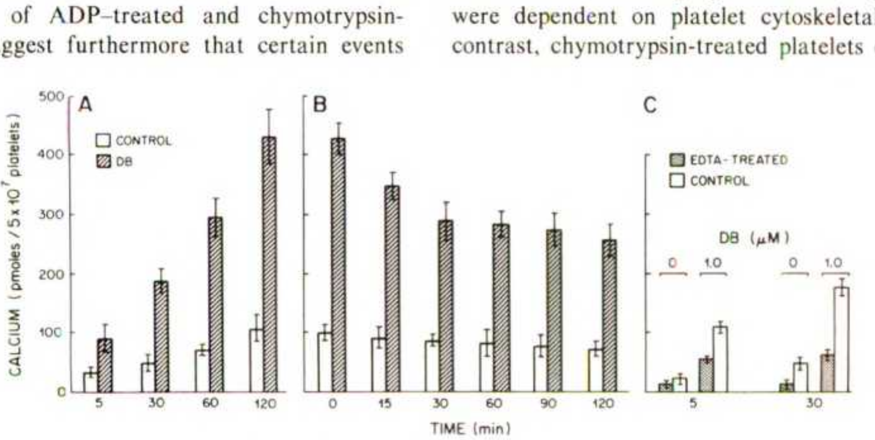

Fig 6. Effect of dibucaine (DB) on platelet calcium homeostasis under steady-state conditions. Platelets, gel-filtered in Ca^{2+} and Mg^{2+}-free HBMT, were incubated for 30 minutes with 0.2 mmol/L of $CaCl_2$, after which a tracer amount of $^{45}Ca^{2+}$ was added together with isotonic saline (□), or 1 mmol/L of DB (□). $^{45}Ca^{2+}$ influx (A) was monitored for two hours after addition of labeled tracer; $^{45}Ca^{2+}$ efflux (B) was assessed after the two-hour Ca^{2+} loading period by washing platelets by centrifugation and resuspension in Mg^{2+}-free HBMT containing 0.2 mg/mL $CaCl_2$ and following changes in platelet-associated Ca^{2+} for an additional two hours. $^{45}Ca^{2+}$ influx following prolonged exposure to platelets to 10 mmol/L of EDTA (□) or the combination of 10 mmol/L of EDTA and 10 mmol/L of $CaCl_2$ (□) is shown in C. Data represent the mean ± SD of five separate experiments.

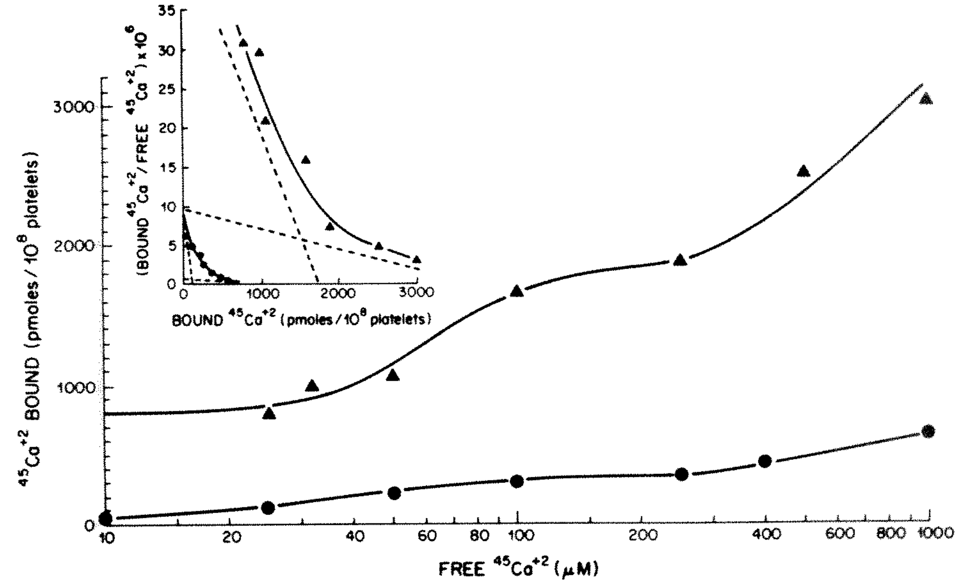

Fig 7. Membrane-associated calcium binding of control (●) and dibucaine (DB)-treated platelets (1 mmol/L for five minutes) (▲) at increasing $^{45}Ca^{2+}$ concentrations. Inset: Scatchard analysis of Ca^{2+} binding. Data represent one of three similar experiments.

require cytoskeleton activation for fibrinogen binding. Because local anesthetics are known to cause depolymerization of microfilaments and microtubules and/or to interfere with cytoskeletal assembly,[13,42] altered GPIIb–IIIa cytoskeleton interactions are likely to be responsible for the different sensitivities of fibrinogen receptors on ADP-treated and chymotrypsin-treated platelets to DB. It is conceivable that the interaction of platelet membrane proteins with membrane-associated cytoskeletal elements alters the exposure of the GPIIb–IIIa complex by modifying its microenvironment and relocating inhibitory membrane constituents that are proteolytically removed by the action of chymotrypsin.[39,43]

Unlike chymotrypsin-treated platelets exposed to cytochalasins, however, chymotrypsin-treated platelets incubated with the local anesthetic DB lost at least some of their ability to bind fibrinogen. This inhibition correlated remarkably well with the DB-induced inhibition of 10E5 binding, a monoclonal antibody that recognizes an epitope on GPIIb and/or GPIIIa when they form a complex, regardless of platelet activation, and suggested that inhibition of fibrinogen binding is mediated in part by alteration of the GPIIb–IIIa complex. SDS-PAGE of DB-treated ^{125}I-labeled platelets, however, revealed no evidence of GPIIb or GPIIIa degradation, and comparison of Triton X-100 lysed platelets by radioelectroimmunoassay indicated that 10E5 recognized similar quantities of GPIIb–IIIa complexes in the presence or absence of DB. Because DB inhibits 10E5 binding to GPIIb–IIIa receptors on intact platelets, however, these data suggest that DB fails to modify GPIIb–IIIa complex integrity directly but may inhibit 10E5 and fibrinogen binding by directly or indirectly altering the conformation of the complex within the platelet membrane or its microenvironment. Moreover, studies examining the effects of DB on ^{45}Ca uptake also suggest that the GPIIb–IIIa complex remains functionally intact following exposure to anesthetic. The GPIIb–IIIa complex was previously implicated in normal Ca^{2+} exchange across the platelet membrane,[24,27] and only prolonged treatment of platelets with EDTA to disrupt this complex resulted in a marked decrease in Ca^{2+} uptake in the present study.

Because local anesthetics have been reported to disturb membrane lipid regions required for membrane protein conformation,[44] and microenvironmental changes surrounding the GPIIb–IIIa complex have been postulated to explain the differing accessibility of this complex to high-mol-wt ligands on activated and nonactivated platelets,[43] changes in platelet membrane lipids were evaluated by examining changes in PF3 availability following platelet incubation with DB. PF3 activity was significantly increased in the presence of DB. Because local anesthetics are potent inhibitors of platelet-dense and α-granule release,[10] changes in PF3 availability were interpreted to reflect changes in the orientation of membrane phospholipids rather than release of factor V.

Exposure of platelets to DB also had a profound effect on platelet Ca^{2+} homeostasis, affecting Ca^{2+} binding, Ca^{2+} exchange, and Ca^{2+} uptake. Ca^{2+} binding to both saturable and nonsaturable membrane binding sites was increased and the Ca^{2+} binding affinity of the saturable sites was decreased. This decreased Ca^{2+} binding affinity may be due to DB-induced changes in the exposure of platelet membrane proteins and/or phospholipids and may contribute to the observed increase in Ca^{2+}exchange across the platelet membrane, since Ca^{2+} binding membrane proteins were previously reported to facilitate plasma membrane Ca^{2+} transport.[27]

DB enhanced total platelet Ca^{2+} uptake nearly fourfold. This increase is mediated in large part by the GPIIb–IIIa complex since disruption of the complex by EDTA-treatment at alkaline pH and 37 °C reduces Ca^{2+} uptake by ~50%. The DB-induced increase in Ca^{2+} uptake appears to activate the platelet Ca^{2+}-dependent protease(s), since the accompanying pattern of proteolysis—especially of ABP and GPIb—resembles that described by others for this protease(s).[9,31] The apparent contradiction between DB-induced increases in Ca^{2+} uptake and the accompanying decrease in platelet aggregation suggests that either the observed increase in Ca^{2+} uptake does not result in similar increases in cytosolic-free Ca^{2+} but rather protein or enzyme-bound Ca^{2+} which may be inactive or inhibitory, or that increased levels of cytosolic free Ca^{2+}, while necessary, are not sufficient for platelet activation. Inhibition of a number of proteins and enzymes including ABP, calmodulin, protein C, and phospholipase A, as well as altered fibrinogen receptor availability, likely result in the observed overall inhibition of platelet cohesion.

Although DB-induced increases in Ca^{2+} uptake correlated directly with degradation of ABP, degradation of GPIb required prolonged platelet incubation with the anesthetic and was usually accompanied by LDH release. Exposure of platelets to at least 0.5 mmol/L of DB for 30 minutes resulted in degradation of GPIb as visualized on PAS-stained polyacrylamide gels of lysed platelets. As described previously,[9] the degradation of both GPIb and ABP was incompletely inhibited by EDTA, indicating that DB-induced release of Ca^{2+} from intracellular stores may also be important, and raising questions concerning the location of the Ca^{2+}-dependent protease(s) (cytoplasmic, membrane bound, or released into the extracellular milieu).

More subtle DB-induced alterations of GPIb could be detected using a monoclonal antibody specific for GPIb (6D1). ^{125}I-labeled 6D1 binding to platelets was reduced following even brief exposure of platelets to low concentrations of DB when GPIb remained intact. These early changes in GPIb are consistent with previously reported decreases in VWF binding to DB-treated platelets stimulated with ristocetin.[9] Such early changes in the functional properties of GPIb may result from anesthetic-induced membrane alterations similar to those postulated to inhibit fibrinogen and 10E5 binding to the platelet membrane GPIIb–IIIa complex.

In conclusion, the data suggest that exposure of platelets to the tertiary amine local anesthetic DB results in several alterations that contribute to the observed inhibition of platelet cohesion: (*a*) Although the platelet membrane GPIIb–IIIa complex appears to remain antigenically intact, and certain complex dependent functions are unaltered in the presence of DB, decreased fibrinogen receptor exposure by traditional agonists including ADP, epinephrine, or thrombin and diminished accessibility of the complex to extracellular ligands such as fibrinogen and the monoclonal antibody 10E5 correlate with DB-induced inhibition of platelet aggregation; and (*b*) increased Ca^{2+} uptake and exchange by DB-treated platelets likely contributes to the observed inhibition of platelet aggregation and activation of the Ca^{2+}-dependent protease(s), leading to degradation of ABP and GPIb which was previously shown to correlate with anesthetic-induced inhibition of ristocetin-mediated platelet agglutination.

ACKNOWLEDGMENT

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Lupus Anticoagulants: Improved Diagnosis With a Kaolin Clotting Time Using Rabbit Brain Phospholipid in Standard and High Concentrations

By Michael H. Rosove, Mohammad Ismail, Brian J. Koziol, Alice Runge, and Carol K. Kasper

We utilized a kaolin-activated partial thromboplastin time (APTT) using rabbit brain phospholipid, in which the capacity of a fourfold increased "high" phospholipid concentration (PC) to normalize the abnormal "standard" PC-APTT in patients with lupus anticoagulants is assessed. This system was also used to measure factors VIIIC, IX, and XI. The tissue thromboplastin inhibition test (TTI), a prothrombin time system in which the activity of a lupus anticoagulant is unmasked by the use of dilute thromboplastin, was simultaneously evaluated. Test sensitivity was defined by results on 31 consecutive patients with standard PC-APTT inhibitors and no bleeding tendency. Specificity was based on 94 patients with various other coagulopathies, including coagulation factor inhibitors, severe congenital factor deficiencies, hepatic insufficiency, and warfarin and heparin treatment. Twenty-one patients with lupus erythematosus and standard PC-APTT results within normal limits were also tested. Sensitivity of the APTT system was superior to that of the TTI (97% v 58%); high PC normalized clotting time ratios and factor levels. Positive results were common with both assays in the group of 20 heparinized patients. The APTT system had superior specificity in remaining cases; there were no positive tests among 74 patients. The lupus erythematosus group had a significant decrease in the clotting time ratio with high PC, indicating that low-level lupus anticoagulants are quite prevalent in this group. The kaolin clotting time using rabbit brain phospholipid in standard and high concentrations is a simple, sensitive, and specific technique for diagnosis of lupus anticoagulants.

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LUPUS anticoagulants are circulating immunoglobulins with activities against phospholipids.[1,2] They are associated with anticardiolipin antibodies,[3] and thrombocytopenia[4]; lupus anticoagulants and anticardiolipin antibodies are strongly associated with thrombosis.[1,3-7] They are found in various clinical settings, most notably idiopathic or drug-induced lupus erythematosus; they may also be isolated findings.[1,2,8-15]

A lupus anticoagulant is suspected when an activated partial thromboplastin time (APTT) inhibitor is found. The APTT is more sensitive than the prothrombin time (PT)[1]; however, the phospholipid source is a crucial determinant of APTT sensitivity.[16,17] Because an APTT inhibitor may reflect the activity of either a lupus anticoagulant or a clotting factor inhibitor, further testing is usually performed for differentiation.

Diagnosis of a lupus anticoagulant has been empirical in the absence of a thoroughly tested and widely accepted diagnostic technique. Spuriously low results in multiple phospholipid-based clotting factor assays that normalize with increasing plasma dilution may be found.[9,16] Demonstration that phospholipid (thromboplastin) source[16] or concentration[9,17-23] is a determinant of assay results provides the most direct evidence: lupus anticoagulants may be unmasked by use of dilute thromboplastin,[9,19] or may be neutralized by phospholipid mixtures insensitive to lupus anticoagulants,[16] concentrated thromboplastin,[17,18] or activated[20,21] or washed, frozen-thawed[22] platelets. Lupus anticoagulants have phospholipid specificity. An IgMλ monoclonal protein with lupus anticoagulant activity was found to have immunologic specificity toward anionic phospholipids including phosphatidylserine, phosphatidylinositol, and phosphatidic acid.[10] Other lupus anticoagulants may be neutralized by phosphatidylserine liposomes, but not by liposomes containing lysophosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine, or phosphatidic acid.[23]

The purpose of the present investigation was development of a simple assay system with a high degree of sensitivity and specificity for lupus anticoagulants that could be performed easily and established in any clinical laboratory. We used a kaolin-activated partial thromboplastin time system using rabbit brain phospholipid, in which the capacity of a "high" phospholipid concentration (PC) to neutralize abnormalities in the "standard" phospholipid concentration test was assessed. We also examine whether lupus anticoagulant neutralization by high PC might provide rapid screening for true factor deficiencies. The tissue thromboplastin inhibition (TTI) test,[9] a PT system using dilute thromboplastin, was simultaneously evaluated for comparative purposes.

METHODS

All studies were performed at 37 °C on frozen-thawed plasmas, prepared from venous blood mixed with 1/10 vol 3.2% sodium citrate and centrifuged (2,500 g, 30 minutes, 4 °C). Plasma pooled from normal subjects served as control. Clotting time ratios were calculated as test divided by control.

Activated Partial Thromboplastin Time System Using Rabbit Brain Thromboplastin in Standard and High Concentrations

Standard PC-APTT. Plasma (0.1 mL) was added to a mixture of kaolin 0.5% in water (0.1 mL) and rabbit brain phospholipid (Platelin, General Diagnostics, Division of Warner-Lambert Co, Morris Plains, NH) (0.1 mL). The phospholipid was used at the manufacturer's recommended concentration; this concentration yielded maximal clotting time shortening in the normal plasma pool. The mixture was incubated (three minutes), $CaCl_2$25 mmol/L (0.1 mL) was then added, and the clotting time was measured (tilt-tube method). The control plasma clotting time was 40.1 ± 5.3 (SD) seconds. Standard PC-APTT assays on 1:1 mixtures of test and control plasma were performed five minutes after mixing.

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High PC-APTT. The procedure was the same as above, except that Platelin was prepared at a fourfold increased concentration by adding one fourth the recommended amount of diluent to the lyophilized phospholipid. The control plasma clotting time was 71.0 ± 11.9 (SD) seconds. Higher PCs gave unacceptably prolonged clotting times in normal plasma and could not be used.

Calculations. The *decrease* in the clotting time ratio caused by concentrated phospholipid, ie, the standard minus the high PC ratio, was calculated. Example: standard PC-APTT = 81.6 s/38.2 s (patient/control) = 2.14. High PC-APTT = 70.2 s/68.8 s = 1.02. Test result = 2.14 − 1.02 = +1.12.

Assays of factors VIIIC, IX, and XI. Assays of factors VIIIC, IX, and XI were performed by slight modification of the APTT. A mixture of equal parts of kaolin and Platelin (either standard or high concentration) (0.2 mL) was added to a mixture of equal parts of congenitally factor-deficient plasma (General Diagnostics) and dilutions (1:10, 1:20, or 1:40 in Owren's buffer) of control reference plasma (Pacific Hemostasis, Bakersfield, Calif) or similarly diluted test plasma (0.2 mL), and incubated (five minutes); $CaCl_2$ 25 mmol/L (0.1 mL) was then added, and the clotting time was determined (tilt-tube method).

PT System Using Thromboplastin in Standard (PT) and Dilute (TTI Test) Concentrations

PT. Plasma (0.1 mL) was added to rabbit brain thromboplastin (Ortho Diagnostic Systems, Inc, Raritan, NJ) (0.2 mL), and the clotting time was determined (Fibrometer method, BBL, division of Becton Dickinson, Cockeysville, Md). The control plasma clotting time was 11.8 ± 0.7 (SD) seconds.

Tissue thromboplastin inhibition test (TTI).[9] Plasma (0.1 mL) was added to Simplastin (rabbit brain and lung thromboplastin, General Diagnostics, diluted 1:500 in NaCl 0.15 mol/L) (0.1 mL) and incubated (five minutes); $CaCl_2$ 25 mmol/L (0.1 mL) was then added, and the clotting time was determined (Fibrometer method). The control plasma clotting time was 85.4 ± 18.4 (SD) seconds.

Calculations. The *increase* in the clotting time ratio by use of dilute thromboplastin, ie, the TTI ratio minus the PT ratio, was calculated. Example: PT = 12.6 s/11.4 s = 1.11. TTI = 138 s/82.2 s = 1.68. Test result = 1.68 − 1.11 = +0.57. The TTI was also evaluated according to the criterion of a positive test proposed by Schleider et al (TTI ratio ≥ 1.3).[9]

Factor II assays. Factor II assays were performed by slight modification of the PT. Congenitally factor-deficient plasma (General Diagnostics) (0.1 mL) and dilutions of control reference plasma (Pacific Hemostasis) or similarly diluted test plasma (0.1 mL) were mixed and incubated (three minutes); rabbit brain thromboplastin (Ortho) (0.2 mL) was then added, and the clotting time was determined (Fibrometer method).

Reagent Lipid Extractions

Total lipid extraction. Solvents used were of high-pressure liquid chromatography (HPLC) grade (Alltech Associated, Inc, Deerfield, Ill) and were redistilled before use to remove nonvolatile residues. Thromboplastin/phospholipid reagents were extracted with hexane:isopropanol (3:2 vol/vol) containing the antioxidant 2,6-di-tert-butyl-*p*-cresol (BHT) at a concentration of 5 mg/dL.[24] Each reagent was extracted four times (25 °C, 20 minutes) with continuous sonication and occasional vortexing, with intervening centrifugation (1,500 g, ten minutes). The fourth extraction yielded an increase of <1% in total lipids. Solvents were removed by evaporation with a rotary vacuum evaporator (Rinco Instrument Co, Inc, model UE-3030 HD). The lipid residue was then resuspended in 3 mL of chloroform for further analyses.

Total lipid determination. Total lipids were determined gravimetrically with a Cahn 25 electrobalance (Cahn Instruments, Inc, Cerritos, Calif) after evaporation to dryness under a stream of nitrogen.

Phospholipid quantitation. Total phospholipid was quantified colorimetrically.[25]

Phospholipid fractionation. Fractionation of phospholipid classes was attempted by two-dimensional thin-layer[26,27] and silicic acid[28] chromatography.

Study Subjects

Normal assay ranges (mean ± 2 SD) were determined by results in 20 healthy, normal subjects.

Test sensitivity. Sensitivity was defined by results on 31 consecutive patients with standard PC-APTT inhibitors and no bleeding tendency. The standard PC-APTT clotting time ratio was corrected in each case by <50% toward 1.00 on mixture with normal plasma [25.0% ± 16.8% (SD)]. The patients had idiopathic (13) or drug-induced (4) lupus erythematosus, chronic idiopathic thrombocytopenic purpura (2), idiopathic warm-type (IgG) immune hemolysis (2), rheumatoid arthritis (1), or no associated condition (9). Fourteen had had one or more thrombotic complications including deep vein thrombosis (7), hepatic vein thrombosis (1), arterial thrombosis (2), transient cerebral ischemia (2), or placental infarction with fetal growth retardation or spontaneous abortion (4). Patients with clinically active, idiopathic lupus erythematosus and standard PC-APTT values within the normal range were also studied (21).

Test specificity. Specificity was determined by studying patients with factor VIIIC anticoagulants (20) [hemophilia A (18), bullous pemphigoid (1), and idiopathic (1), with a median Bethesda U titer[29] of 20 U (range 1.7 to 300 U)]; a congenitally factor XI-deficient patient with a 3 Bethesda U inhibitor; patients with severe congenital factor deficiencies (25) (standard PC-APTT clotting time ratio was corrected in each case by ≥80% toward 1.00 upon mixture with normal plasma [91.5% ± 6.2% (SD)]); and patients with hepatic insufficiency and a PT ratio ≥1.3 (8), or receiving warfarin (20) or heparin (20) treatment.

RESULTS

APTT System Results

Results in various study groups. Clotting time ratios (mean ± SD) obtained are shown in Table 1. High PC decreased the clotting time ratio in the "test sensitivity" (lupus anticoagulant) group such that it became indistinguishable from the normal subject group (1.06 ± 0.21 *v* 1.08 ± 0.21), whereas remaining groups with increased standard PC-APTT clotting time ratios remained increased in the high PC-APTT. The lupus erythematosus group had a mean standard PC-APTT not significantly different from the normal subject group (P = NS) (Table 1), yet high PC caused a significantly greater decrease in the clotting time ratio [−0.04 ± 0.16 (SD) *v* −0.13 ± 0.15, $t = 2.2$, $P < .05$].

Individual patient results. Data obtained in individual subjects are shown in Figs 1 and 2. Thirty of 31 "test sensitivity" (lupus anticoagulant) patients had a significant decrease in clotting time ratio (≥0.17 ratio units). Some lupus erythematosus patients with normal standard PC-APTT had significant decreases. Significant decreases were noted occasionally in factor VIIIC anticoagulant patients who were readily distinguishable from "test sensitivity"

Table 1. Clotting Time Ratios Obtained in Various Study Groups

Study Group	N	Standard PC-APTT	High PC-APTT	PT	TTI
Normal subjects	20	0.95 ± 0.10 (1.00 ± 0.05)*	1.08 ± 0.21	0.97 ± 0.08	1.17 ± 0.20
Lupus anticoagulants	31	1.74 ± 0.52 (1.53 ± 0.36)	1.06 ± 0.21	1.14 ± 0.16	1.77 ± 0.53
Lupus erythematosus with normal standard PC-APTT	21	0.92 ± 0.12 (0.96 ± 0.07)	0.96 ± 0.18	1.01 ± 0.08	1.05 ± 0.22
Factor VIIIC anticoagulants	21	3.17 ± 0.61 (1.90 ± 0.18)	3.31 ± 0.74	1.06 ± 0.09	1.26 ± 0.31
Factor XI anticoagulant	1	3.31 (3.30)	4.76	1.04	0.95
Severe congenital deficiency					
Factor VIIIC	9	3.43 ± 0.54 (1.20 ± 0.08)	3.89 ± 0.47	1.09 ± 0.07	1.66 ± 0.04
Factor IX	11	3.16 ± 2.05 (1.13 ± 0.10)	4.33 ± 2.38	1.10 ± 0.06	1.86 ± 0.52
Factor XI	2	3.75, 1.74 (1.14, 1.15)	4.34, 3.68	0.91, 1.12	2.08, 0.95
Factor VII	2	0.92, 0.97 (0.94, 0.97)	1.02, 1.00	1.80, 2.73	2.04, 1.34
Factor V	1	4.80 (1.17)	4.62	2.83	3.40
Hepatic insufficiency	8	1.51 ± 0.37 (1.10 ± 0.11)	2.18 ± 0.61	1.63 ± 0.22	1.56 ± 0.70
Warfarin patients	20	1.44 ± 0.24 (1.10 ± 0.80)	1.70 ± 0.46	1.83 ± 0.32	1.52 ± 0.54
Heparin patients	20	2.64 ± 1.27 (1.72 ± 1.06)	1.78 ± 0.40	1.14 ± 0.11	2.87 ± 1.64

PC-APTT, phospholipid concentration–activated partial thromboplastin time; TTI, tissue thromboblastin inhibition.

Ratios are mean ± SD.

*In 1:1 mixture with normal plasma (five-minute incubation).

(lupus anticoagulant) patients by marked residual abnormalities in the high PC-APTT in all 20 cases, whereas all 31 "test sensitivity" (lupus anticoagulant) patients were within the normal range [1.08 ± 0.42 (2 SD)] (Fig 2). Decreases of ≥0.17 units were common in heparinized patients (Fig 1).

Test interpretation. Segregation of "test sensitivity" (lupus anticoagulant) patients from remaining patients could be made with a high degree of accuracy (Table 2) by using both the clotting time ratio decrease and the high PC-APTT result. The test was considered "positive" and indicative of a lupus anticoagulant if the standard PC-APTT was prolonged, and high PC reduced the clotting time ratio both by ≥0.17 units *and* into the normal range. (Neither criterion alone was as accurate: some "test specificity" patients had significant clotting time ratio decreases only; others without a significant clotting time ratio decrease fell within the normal range in the high PC test only because the high PC normal range was broader.) The test was "weakly positive" if the standard PC-APTT was normal, but high PC reduced the clotting time ratio by ≥0.17 units. The test was "indeterminate" if the standard PC-APTT was prolonged, and the reduction was ≥0.17 units, but not into the normal range. It was "negative" if the clotting time ratio was not ≥0.17 units.

Factor VIIIC, IX, and XI levels. High PC raised the apparent factor VIIIC level in all seven randomly selected "test sensitivity" (lupus anticoagulant) plasmas tested at 1:10 dilution into the normal range (0.5 to 2.0 U/mL); it had much less but possibly some corrective effect in factor VIIIC anticoagulant plasmas (Fig 3). Similar results were noted in factor IX and XI assays (Table 3).

PT/TTI System Results

Results in various study groups and individual patients. Results are shown in Table 1 and Fig 4. Eighteen of 31 (58%) "test sensitivity" (lupus anticoagulant) patients

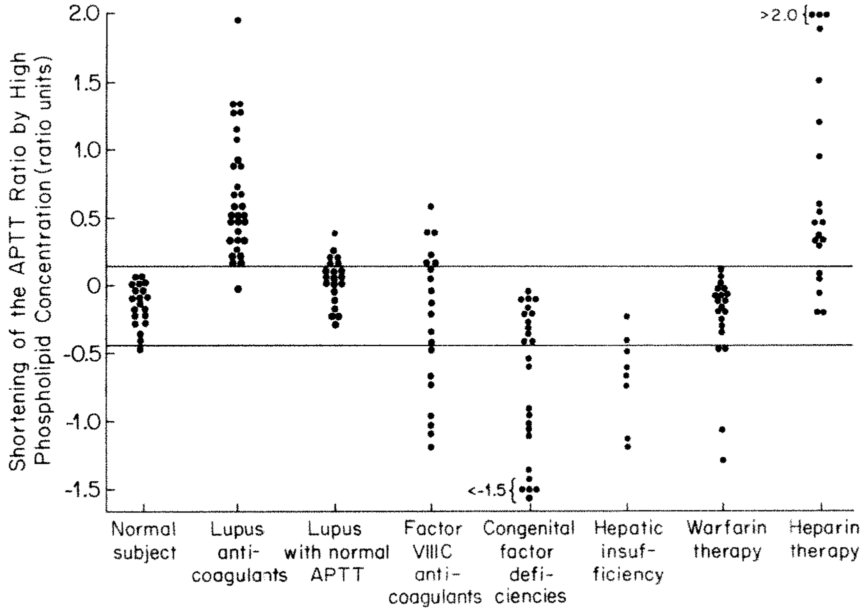

Fig 1. Activated partial thromboplastin time (APTT) system results in individual patients. The horizontal lines delineate the normal range (−0.43 to +0.17) based on the mean (±2 SD) of normal subjects.

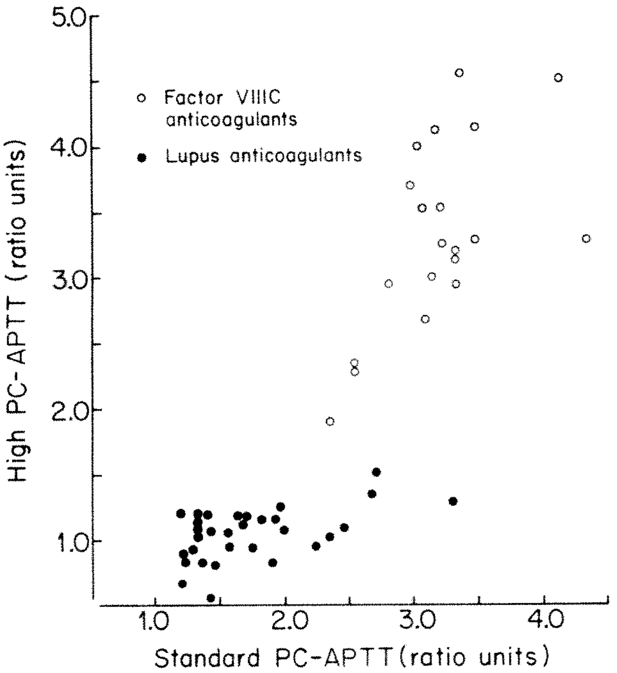

Fig 2. High and standard phospholipid concentration-activated partial thromblastin time (PC-APTT) results in "test sensitivity" (lupus anticoagulant) and factor VIIIC anticoagulant patients.

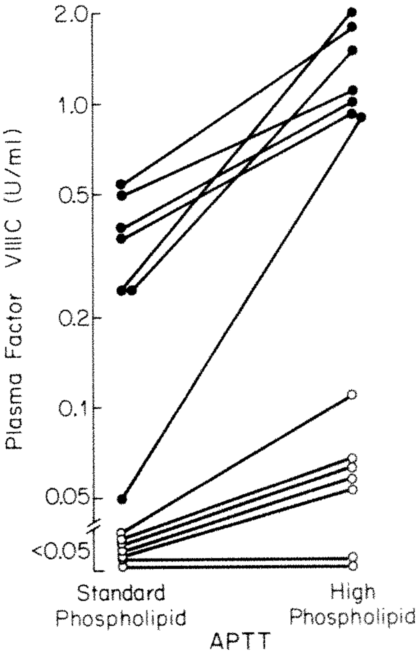

Fig 3. Effect of high phospholipid concentration (PC) on apparent factor VIIIC levels in "test sensitivity" (lupus anticoagulant) and factor VIIIC anticoagulant patients.

Table 2. Interpretation of APTT Assay Results

Study Group	N	Qualitative Test Results*			
		Positive	Weakly Positive	Indeterminate	Negative
Standard PC-APTT inhibitors and no bleeding tendency	31	30	—	0	1
Factor VIIIC anticoagulants	20	0	—	6	14
Factor XI anticoagulant	1	0	—	0	1
Congenital factor deficiencies	25	0	—	0	25
Hepatic insufficiency	8	0	—	0	8
Warfarin patients	20	0	—	0	20
Heparin patients	20	3	—	12	5
Lupus erythematosus with normal standard PC-APTT	21	—	6	0	15

Abbreviations as in Table 1.

*Positive: standard PC-APTT abnormal; high PC reduces clotting time ratio ≥0.17 U and into normal range. Weakly positive: standard PC-APTT normal; high PC reduces clotting time ratio ≥0.17 U. Indeterminate: standard PC-APTT abnormal; high PC reduces clotting time ratio ≥0.17 U but not into normal range. Negative: high PC does not reduce clotting time ratio ≥0.17 U.

Table 3. Effect of Standard v High PC on Apparent Clotting Factor Levels in APTT-Based Assays

Study Group	Plasma Dilution	Normal Subjects		Lupus Anticoagulant Patients		Factor VIIIC Anticoagulant Patients	
		Standard	High	Standard	High	Standard	High
Factor VIIIC	1:10	0.90 ± 0.29	0.88 ± 0.13	0.34 ± 0.17	1.32 ± 0.45	0.03 ± 0.01	0.05 ± 0.03
	1:20	0.90 ± 0.28	0.94 ± 0.31	0.55 ± 0.34	1.55 ± 0.58	0.05 ± 0.04	0.08 ± 0.05
	1:40	0.85 ± 0.22	1.07 ± 0.44	0.71 ± 0.40	1.85 ± 0.67	0.09 ± 0.05	0.14 ± 0.07
Factor IX	1:10	0.93 ± 0.20	0.76 ± 0.22	0.20 ± 0.12	0.90 ± 0.16	0.65 ± 0.26	1.10 ± 0.63
	1:20	0.91 ± 0.28	0.77 ± 0.31	0.28 ± 0.26	1.07 ± 0.17	0.74 ± 0.29	1.11 ± 0.47
	1:40	0.86 ± 0.29	0.77 ± 0.32	0.40 ± 0.28	1.38 ± 0.28	0.87 ± 0.36	1.23 ± 0.51
Factor XI	1:10	0.95 ± 0.13	0.77 ± 0.13	0.47 ± 0.32	1.72 ± 1.03	0.97 ± 0.28	1.32 ± 0.63
	1:20	0.81 ± 0.21	0.71 ± 0.13	0.65 ± 0.38	1.69 ± 0.67	1.33 ± 1.01	1.52 ± 0.97
	1:40	0.81 ± 0.21	0.64 ± 0.09	0.92 ± 0.54	1.60 ± 0.55	1.25 ± 0.55	1.34 ± 0.73

Clotting factor levels in U/mL; values are mean ± SD; seven randomly selected subjects in each group. PC, phospholipid concentration.

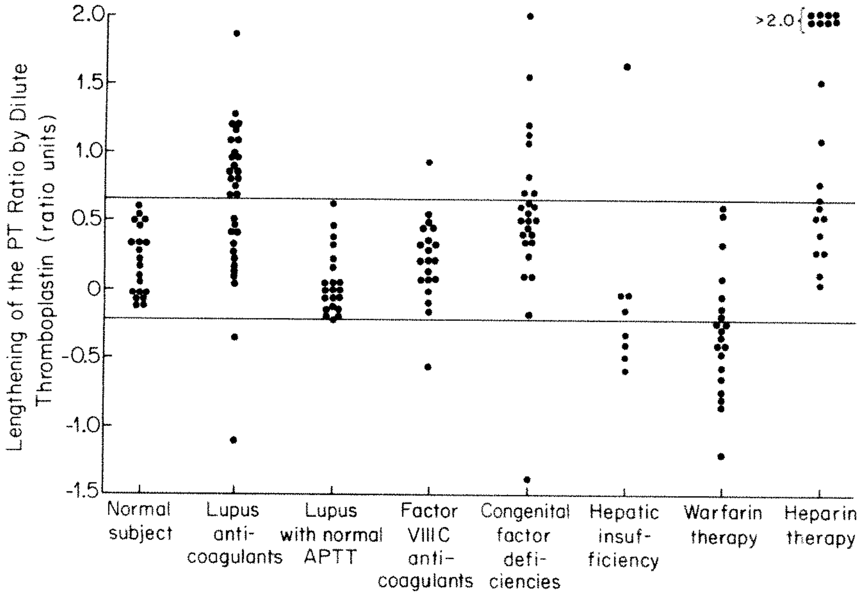

Fig 4. Prothrombin time/tissue thromboplastin inhibition (PT/TTI) system results in individual patients. The horizontal lines delineate the normal range (−0.24 to +0.66) based on the mean (±2 SD) of normal subjects.

had positive tests (≥0.66 ratio unit increase by dilute thromboplastin). The TTI did not unmask abnormalities in the lupus erythematosus group with normal standard PC-APTT as compared with normal subjects [+0.04 ± 0.22 (SD) *v* +0.21 ± 0.22, P = NS]. Positive results were common in congenitally factor-deficient and heparinized patients. The TTI was nonspecific when a ratio of ≥1.3 was used as the criterion of a positive test[9] (Table 4).

Factor II levels. The PT ratio was ≥1.3 in three "test sensitivity" (lupus anticoagulant) patients. Two had factor II levels of 76% and 83%; the third patient would not volunteer additional blood.

Table 4. Frequency of Positive TTI Tests by Criterion of TTI Ratio ≥1.3

Group	Positive Tests/ Total Patients (%)
Normal subjects	6/20 (30)
Lupus anticoagulants	26/31 (84)
Lupus erythematosus with normal standard PC-APTT	3/21 (14)
Clotting factor inhibitors	9/21 (43)
Congenital clotting factor deficiencies	24/25 (96)
Hepatic insufficiency	6/8 (75)
Warfarin patients	11/20 (55)
Heparin patients	17/20 (85)

Abbreviations as in Table 1.

Table 5. Lipid and Phospholipid Present in Clotting Assays

	Amount of Material in Each Assay (mg/mL of Plasma)		
Assay*	Total	Lipid	Phospholipid
Prothrombin time	19.2	4.70	0.93
TTI test	0.023	0.016	0.0006
Standard PC-APTT	14.0	9.12	0.41
High PC-APTT	56.0	36.5	1.66

*Thromboplastin/phospholipid sources given in Materials and Methods section. Abbreviations as in Table 1.

Reagent Lipid Analyses

Compositions of the thromboplastin/phospholipid reagents are shown in Table 5. Attempts to separate and quantify individual phospholipid classes were complicated by interference caused by the heterogeneity and complexity of the lipids in the reagents and the small fraction of phospholipid present.

DISCUSSION

Among 31 patients with standard PC-APTT inhibitors and no bleeding tendency, the high PC-APTT identified 30 patients with positive results (97% sensitivity). These patients appear to have phospholipid inhibitors (lupus anticoagulants) because the inhibitors were overcome by boosting the phospholipid concentration of the assay system. Nearly all patients with APTT inhibitors and no bleeding tendency thus appear to have lupus anticoagulants; specific clotting factor inhibitors in such patients appear to be quite uncommon.

High PC decreased the clotting time ratio significantly in some factor VIIIC anticoagulant patients and also increased the apparent factor VIIIC level in some patients. These patients were distinguishable from lupus anticoagulant patients in each case by their uncorrected high PC-APTT results; the residual high PC-APTT abnormalities were likely due to factor VIIIC deficiency. It is nevertheless possible that some factor VIIIC anticoagulants may also possess lupus anticoagulant-like behavior, or that some may coexist with lupus anticoagulants. It is unlikely that high PC activated factor VII with a resulting intrinsic cascade bypass, because similar clotting time ratio decreases among the 22 patients with congenital deficiencies of factors VIIIC, IX, and XI were not observed. False-positive and indeterminate results were frequently encountered among heparinized patients; testing in this group is thus not advisable. No positive results were obtained among 74 patients with clotting factor inhibitors, congenital factor deficiencies, hepatic insufficiency, or warfarin treatment.

The APTT system with rabbit brain phospholipid thus has a high degree of sensitivity and specificity for lupus anticoagulants, provided that heparin is not present, and appears to be a useful addition to clinical diagnosis. High PC normalized factor VIIIC, IX, and XI levels in the presence of lupus anticoagulants; clotting time normalization by high PC thus appears to obviate the need to assay factors VIIIC, IX, and XI for purposes of excluding true deficiency.

High PC significantly reduced the clotting time ratio in the group of 21 patients with active idiopathic lupus erythematosus and standard PC-APTT results within the normal range. An often-quoted figure for the incidence of lupus anticoagulants in lupus erythematosus is 5% to 10%. The reported range is broad, however, ranging from 0.4% to 65%,[1] likely reflecting differences in test reagents.[1,16,17] Our results with a sensitive system suggest that the 5% to 10% figure is an underestimate and that low level lupus anticoagulants are quite prevalent in lupus erythematosus. Anticardiolipin antibodies are found by radioimmunoassay in ~50% of this patient group.[3]

The APTT system we have described is a rabbit brain neutralization procedure (RBNP); as such, it shares with other systems[17,18,20-22] the principle that lupus anticoagulants can be overcome or bypassed by providing an additional source of phospholipid. Veltkamp et al[17] first described this approach in 1973; it has since been used with modifications by others.[18,20-22] Neutralization assays may be viewed as the reverse of dilute thromboplastin assays,[9,19] in which lupus anticoagulants are unmasked by removing phospholipid from the system. There has been little clinical application of neutralization assays with the exception of the "platelet neutralization procedure (PNP)" described by Triplett et al.[22] In the PNP, washed, frozen-thawed platelets (2 to 3×10^8 platelets/mL of plasma) are added to an APTT system to overcome lupus anticoagulants. The PNP distinguishes patients with lupus anticoagulants from patients with clotting factor inhibitors; clotting time correction in lupus anticoagulant patients is generally partial.[22,30] In the RBNP, all 30 clotting time corrections in lupus anticoagulant patients corrected to the normal range. The difference in rabbit brain PC between standard and high PC tests was 1.25 mg/mL of plasma; platelets added to the PNP system contained 0.06 to 0.09 mg phospholipid/mL of plasma, a figure derived from studies of platelet lipid composition.[31,32] This difference may well be significant, but no definitive comment can be offered without quantification of specific phospholipids.

Neutralization systems appear to be an improvement over the TTI. The originally proposed criterion of a positive TTI test (ratio ≥1.3)[9] has been widely applied. By this definition, Triplett showed nonspecificity in patients with clotting factor inhibitors or in those receiving heparin; we found the test to be sensitive (84%) but nonspecific, with high false-positive rates in every test specificity group (45% to 96%) including normal subjects (30%). By our definition of a positive test (spread of the TTI and PT ratios exceeding the mean + 2 SD of normal subjects, equating to a TTI ratio ≥1.63), false-positive results were less prevalent, at the expense of sensitivity (58%).

The TTI was insensitive to some lupus anticoagulants even though the total PC was only 0.0006 mg/mL of plasma. Recently, phosphatidylserine 24 μmol/L, equating to ~0.03 mg/mL of plasma in the test system, was found to cause significant but variable clotting time shortening in the presence of lupus anticoagulants.[23] Lupus anticoagulants thus may vary broadly in susceptibility to phosphatidylserine over at least a 50-fold range of concentrations; alternatively, other lipid(s) and phospholipid(s), or their specific configurations, may also be important. That a lupus anticoagulant may have specificity for several phospholipids has already been demonstrated.[10]

Specific reagents, for example, liposomes of known composition, may someday provide the basis of new lupus anticoagulant assays. The variables inherent in animal or plant tissues and their preparation might then be avoided, and thus, too, the vagaries of the PT and APTT as we now know them. In the meantime, the use of a widely available, sensitive reagent seems the best option. The sytem we describe should be valuable for some time, pending development of the next "generation" of test systems.

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Monocyte Nonspecific Esterase: Purification and Subunit Structure

By Joseph Yourno

Monocyte nonspecific esterase has been purified from cultured cells of the acute myeloid leukemia cell line, ML-1. The purified enzyme shows the characteristic properties of the monocyte neutral serine carboxyl esterase, with high sensitivity to organophosphorus inhibitors and sodium fluoride inhibitor. The enzyme is a membrane protein which in the native state exists as a monomer of a mol wt of ~68,000 and a trimer of mol wt 205,000. These forms exhibit a complex pattern of dissociation and reassociation based on apparent noncovalent binding of subunits. The delipidated dissociated enzyme runs as a single protein chain of a mol wt of ~62,000 on sodium dodecyl sulfate (SDS) gel electrophoresis. The relation of the subunits to monocyte isoenzymes seen on isoelectric focusing (IEF) and polyacrylamide gel electrophoresis at pH 9.5 (pH 9.5 PAGE) of cell extracts is demonstrated. Availability of purified enzyme allows development of monoclonal antibodies and analysis of myeloid differentiation. In addition, the substrate specificity and function of the purified monocyte ectoenzyme are being examined.

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MONOCYTE nonspecific esterase continues to be a key cytochemical marker in the classification of acute leukemias as recommended by the French-American-British (FAB) study group on acute leukemias.[1] In addition, this enzyme marker is widely used in studies of myeloid and monocyte/macrophage differentiation.[2] The high sensitivity of cytochemical monocyte esterase to organophosphorus inhibitors[3] reveals that the enzyme belongs to the special group of carboxyl esterases that use a nucleophilic serine residue at the active site for hydrolysis.[4] Electron microscopy suggests that the monocyte esterase is a membrane ectoenzyme,[5-8] but the physiological role of this unique enzyme remains unknown. Although a wealth of studies has accumulated over the years on the cytochemistry[6,9,10] and more recently the isoenzymology[11-14] of monocyte esterase, almost nothing has been reported on the purification and properties of the enzyme at the level of classical enzymology. Availability of purified enzyme is key to the production of monoclonal antibodies to monocyte esterase for improved subtyping of acute myeloid leukemias and for studies of monocyte differentiation. Furthermore, a description of the basic molecular and enzymologic properties of the enzyme is necessary to elucidate its molecular biology and its role in monocyte/macrophage physiology. This report describes the purification, subunit structure, and enzymology of the monocyte esterase from cultured cells of the acute myeloid leukemia cell line, ML-1.

MATERIALS AND METHODS

Source and Characteristics of Cultured ML-1 Cells

Acute myeloid leukemia (AML) cell line ML-1[15] was isolated from the peripheral blood of a 24-year-old man with AML by M.S. Lok and generously furnished to us by Dr J. Minowada of Roswell Park Memorial Institute. Morphologically, ML-1 cells consist of a relatively monomorphic population of blasts, 15 to 25 μm in diameter, which on Wright's stain show a moderate nuclear/cytoplasmic ratio and agranular to finely granular basophilic cytoplasm. Cytochemically, >90% of ML-1 cells are strongly positive for fluoride-sensitive nonspecific esterase, whereas 30% to 80% are positive for myeloperoxidase, Sudan Black B reactivity, and naphthol-ASD-chloro-acetate esterase (J. Yourno et al, manuscript in preparation). The ML-1 cell line therefore tests as myelomonocytic (AML-M4). Because ML-1 produces very high levels of monocyte esterase, it is an ideal source of enzyme for purification and molecular analysis.

Growth and Harvest of Cultured ML-1 Cells

Cultures of ML-1 were grown to plateau phase (20 to 25×10^5 cells/mL) in sealed 2L roller bottles containing 500 mL of RPMI 1640 growth medium with 10% fetal calf serum (FCS) and 50 μg of gentamycin sulfate per milliliter. Pooled cultures of $\sim 1.4 \times 10^{10}$ cells in 1.6 L of growth medium were harvested, washed, and stored at −60 °C as previously described.[16]

Preparation of Crude Extracts

Cell suspensions totaling 600 to 800 mL, containing $\sim 1.6 \times 10^{11}$ cells in 20 mmol/L of 2[*N*-morpholino]ethane sulfonic acid (MES) buffer, pH 6.4, 20% glycerol, were thawed on ice. Triton X-100 (Sigma, St Louis) was added to a final concentration of 1%; the suspension was kept on ice for 30 to 60 minutes and stirred occasionally. The detergent-treated suspension was centrifuged at 16,000 g for 15 minutes, and the milky supernatant was taken as the crude extract. The pellet was resuspended in 50 mL of buffer, recentrifuged as above, and the wash was combined with the crude extract. The crude extract yielded about 1 to 1.5×10^6 mU of ANB esterase and 3 to 4.5×10^5 mU of ANA esterase.

Buffers

Buffer A consisted of 10 mmol/L of MES, pH 6.4, containing 20% glycerol. Buffer B consisted of buffer A with 33 mmol/L of NaCl; buffer C consisted of 1 mmol/L of KH_2PO_4, pH 6.8; buffer D consisted of 500 mmol/L of KH_2PO_4, pH 6.8; and AS consisted of saturated $(NH_4)_2SO_4$ containing 10 mmol/L of MES, pH 6.4.

Chromatography Materials

Diethyaminoethanol (DEAE)-cellulose (DH-52) was purchased from Whatman, Clifton, NJ; Sephadex G-200 from Pharmacia, Piscataway, NJ; and hydroxyapatite-agarose Ultrogel from LKB, Rockville, Md. Mol wt standards for gel-filtration chromatography (Sigma) included blue dextran, catalase, bovine serum albumin, and cytochrome C.

PAGE and IEF of Nonspecific Esterase

PAGE at pH 9.5 (pH 9.5 PAGE) and IEF were performed as previously described.[11,13] Unless otherwise specified, all gels were run with a standard pH gradient from 3.5 to 9.0. ANA substrate was used to develop gels, since this stains monocyte esterases as well as

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ANB substrate does and reveals other nonspecific esterases that are not stained by ANB.[11-13] SDS-PAGE was performed according to Laemmli.[17] SDS was purchased from Bio-Rad, Richmond, Calif. Water-dialyzed specimens containing ~5 μg of protein were run. The specimens were made to 10% in methanol and extracted three times with an equal volume of heptane. The extracted aqueous layer was dried under an air stream, and the residue was dissolved in dissociating buffer.[17] Mol wt standards for SDS-PAGE were purchased from Pharmacia.

Enzyme Assays and Enzymological Studies

Nonspecific esterase activity was assayed by a kinetic ultraviolet (UV) method.[18] Substrates included α-naphthyl acetate (ANA) and α-naphthyl butyrate (ANB). Inhibitor studies with sodium fluoride (NaF) and diisopropylfluorophosphate (DFP) were performed with substrate mixture made to the desired concentration of inhibitor from stock solutions of these (400 mmol/L of NaF in buffer A; 1 mol/L of DFP in isopropanol). Protein was determined according to Bradford as previously described.[18]

Enzyme Purification

Ion-exchange chromatography. The detergent-treated crude extract in buffer A was applied to a DH-52 column equilibrated in the same buffer. The flow-through fraction was collected, and the bound fraction was eluted with a NaCl gradient in buffer A. Fractions collected were assayed for ANB esterase activity.

Preparative gel-filtration chromatography. The major esterase peak from the ion-exchange chromatography was pooled and dialyzed *v* AS solution. The resulting precipitate was collected by ultrafiltration through a glass fiber filter (Whatman GF/C). The collected protein was dissolved in 10 mL of buffer B and centrifuged at 16,000 g for ten minutes. The clarified supernatant and two washes each of 2 mL of buffer B were combined. Blue dextran marker was added to the enzyme solution to a final concentration of 1 mg/mL. The solution was subjected to gel-filtration chromatography through a 4.2 × 90 cm Sephadex G-200 column in buffer B, and 10-mL fractions were collected. Fraction volumes were measured to establish elution volume (V_e). The Blue dextran peak was determined spectrophotometrically at 625 mμ to establish the void volume (V_0). Fractions were then analyzed for ANB esterase activity. Eluted enzyme was combined into three pools according to V_e, and the pools were dialyzed *v* AS solution. The G-200 column was calibrated by rechromatography with a mixture of mol wt standards: Blue dextran, mol wt 2×10^6, 1 mg/mL; catalase, effective mol wt 195,000, 1 mg/mL; bovine serum albumin, mol wt 68,000, 10 mg/mL; and cytochrome c, mol wt 12,500, 5 mg/mL. Blue dextran was measured as described above; catalase, albumin, and cytochrome C were measured by standard spectrophotometric methods.[19]

Hydroxyapatite adsorption chromatography. The major nonspecific esterase peak from the G-200 gel-filtration chromatography, GII, average mol wt 205,000, was subjected to adsorption chromatography. The GII peak in buffer C was applied to a 5.0 × 4.0 cm hydroxyapatite column equilibrated in the same buffer. The flow-through fraction was collected, and bound material was eluted with a linear KH_2PO_4 gradient. Fractions collected were assayed for ANB esterase activity. Enzyme peak fractions were pooled and dialyzed *v* AS solution. Rechromatography of hydroxyapatite peaks was performed similarly. Column dimensions and gradient volumes were scaled down to maintain proportionality. Fractions of 1.25 mL were collected, assayed for ANB esterase and protein, pooled, and dialyzed *v* AS solution.

Analytical Gel-Filtration Chromatography

Conditions were as described above for preparative Sephadex G-200 chromatography except that 1.0-mL specimens containing 400 to 2×10^5 mU ANB esterase were run. Column dimensions were 1.6 × 90 cm. Fractions of 1.0 mL were collected and assayed for ANB esterase.

Analytical Hydroxyapatite Adsorption Chromatography

Conditions were as described above for preparative chromatography. Crude extract specimens of 1.0 mL containing 1,600 to 2,000 mU of ANB esterase were run on 1.5 × 6.0 cm columns, and fractions of 1.0 mL were collected.

RESULTS

Crude extract nonspecific esterase of cultured ML-1 cells (AML-M4) averaged about 1,800 mU/mL with ANB substrate and 500 mU/mL with ANA substrate. This activity equals that of the most active normal donor monocyte and acute monocytic leukemia (AML-M5) preparations we have seen.

Analytical Chromatography

When the crude extract was subjected to analytical gel-filtration chromatography on Sephadex G-200, an elution pattern characteristic of monocyte esterase was found. This pattern consisted of a major enzyme peak, GII, of a mol wt of ~205,000, and a subpeak, GIII, of a mol wt of ~68,000. A minor peak, GI, at the void volume, consisted of presumed aggregates of high mol wt (Fig 1). Recovery of enzyme was ~90% of applied activity. On pH 9.5 PAGE, the crude extract showed a zymogram similar to that of normal monocytes,[11] consisting of: (a) an intense slower form, AB-2; and (b) a faster, less prominent form, AB-1. On pH 9.5 PAGE, the GII peak showed both forms at approximately equal intensity; the GIII peak enzyme showed predominantly the faster form, AB-1 (Fig 1, inset). These results suggest that the native enzyme, extracted from the cell membrane with nonionic detergent, consists of a moiety of a mol wt of ~68,000, AB-1, and a moiety of a mol wt of ~205,000, AB-2. Apparent enrichment of AB-1 in the GII peak suggests that this form is generated from AB-2.

On IEF, the crude extract showed the characteristic series of closely spaced monocyte esterase bands[12,13] in the pI range 5.8 to 6.3; the more anionic forms were more prominent (Fig 2). On the same system, the GII peak showed a similar series of bands but with increased intensity of the less anionic forms; the GIII peak showed the less anionic forms predominantly. These results suggest that in unfractionated cell extracts AB-1 consists of two or more forms with a pI of 6.2 to 6.3, whereas AB-2 consists of multiple forms with slightly lower pI values.

The crude extract of ML-1 was also examined by analytical adsorption chromatography on hydroxyapatite. Two peaks of esterase activity were obtained: a minor peak, HAI, eluting at about 100 mmol/L of phosphate; a major peak, HAII, eluting at about 200 mmol/L of phosphate (Fig 3). These peaks were also examined on pH 9.5 PAGE (Fig 3,

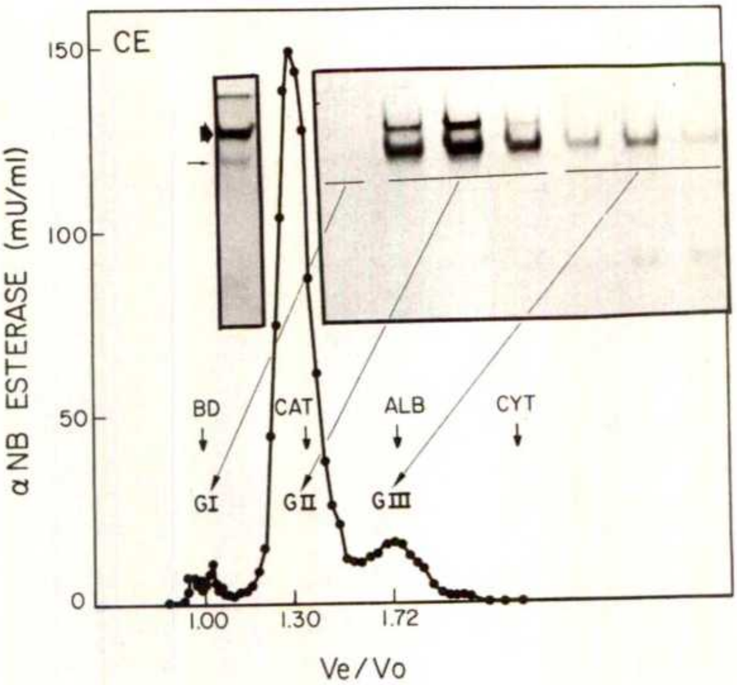


Fig 1. Analytical gel-filtration chromatography of ML-1 crude extract esterase. A 1-mL aliquot of crude extract containing 1,800 mU of ANB esterase in buffer B with 1 mg/mL Blue dextran was chromatographed on a 1.6 × 90 cm Sephadex G-200 column in buffer B; 1-mL fractions were collected, measured volumetrically, and assayed for ANB esterase. The column was next calibrated with a mixture of mol wt markers: Blue dextran (BD) (mol wt 2 × 10^6); catalase (CAT) (mol wt 195,000); bovine serum albumin (ALB) (mol wt 68,000); and cytochrome C (CYT) (mol wt 12,500). Arrows indicate midpoint of marker peaks. Elution profiles are expressed as ratio of peak elution volume, V_e, to BD elution volume, V_o, or void volume. The GI peak enzyme elutes near the void volume ($V_e/V_o = 1.00$), GII peak enzyme at mol wt ~205,000 ($V_e/V_o = 1.30$), and GIII peak enzyme at mol wt ~68,000 ($V_e/V_o = 1.72$). Inset: pH 9.5 polyacrylamide gel electrophoresis zymograms of monocyte esterase fractions from gel-filtration chromatography, ANA-Fast Blue BB stain. From left to right: unfractionated crude extract; peak GI; peak GII-leading edge, peak, trailing edge; peak GIII-leading edge, peak, trailing edge. Bold arrow indicates slower AB-2 form; fine arrow indicates faster AB-1 form. Enzyme from Sephadex peaks was applied to gel at a standardized volume of 60 µL for direct comparison. The amount of AB-1 appears increased in G200 fractions as compared with crude extract, and AB-1 is the major form in peak GIII. Also seen faintly in the crude extract are the fast migrating acetyl esterases A1 and A2, closely appositioned. These isoenzymes were found in all blood cells examined, ie, genuinely nonspecific.[11] Because the mol wts of A1 and A2 are similar to the mol wt of AB-1, these forms elute with the GIII peak in gel-filtration chromatography of crude extract. Normal monocyte esterase gives a similar pattern of peaks on gel filtration chromatography (data not shown).

inset) and IEF (Fig 2) *v* unfractionated crude extract control enzyme. The HAI peak was seen to correspond to the AB-1 form with highest pI values; the HAII peak showed a mixture of the AB-1 and AB-2 forms, covering the entire pI range seen with unfractionated enzyme. Again the data suggest generation of AB-1 from AB-2 following the chromatography step.

Preparative Chromatography

When the crude extract of ML-1 was subjected to ion-exchange chromatography on DEAE-cellulose, a major peak, DHI, was eluted at 50 mmol/L of NaCl (data not shown). This elution profile is similar to that of normal donor monocyte esterase.[20] This peak contained 90% of recovered esterase and showed a typical monocyte zymogram on pH 9.5 PAGE and IEF (data not shown).

The concentrated enzyme from the DHI peak was next subjected to preparative gel filtration on Sephadex G-200, yielding an elution pattern resembling that given by crude extract of ML-1 and normal monocytes (data not shown). Likewise, on pH 9.5 PAGE and IEF, the GI, GII, and GIII peak enzymes resembled those of the corresponding peaks obtained directly from the crude extract (data not shown).

The Sephadex G-200 major peak GII enzyme was next subjected to adsorption chromatography on hydroxyapatite-agarose. The enzyme was eluted in the characteristic two peaks: HAI and HAII (Fig 4). On SDS-PAGE, the delipidated, dissociated enzyme from each peak was found to be associated with a prominent single protein chain of a mol wt of ~62,000 (Fig 4, inset). The intensity of the protein band was proportional to the size of each HA peak. The HAII peak was nearly homogeneous with respect to this protein. These results clearly suggest that the fundamental subunit of monocyte esterase is a single protein chain of a mol wt of ~68,000 which, when delipidated, has a mol wt of ~62,000. The proportion of carbohydrate in this subunit remains to be determined.

The pooled enzyme peaks were rechromatographed on hydroxyapatite-agarose. The HAI peak consistently generated an HAII peak of about one-half its size in several runs

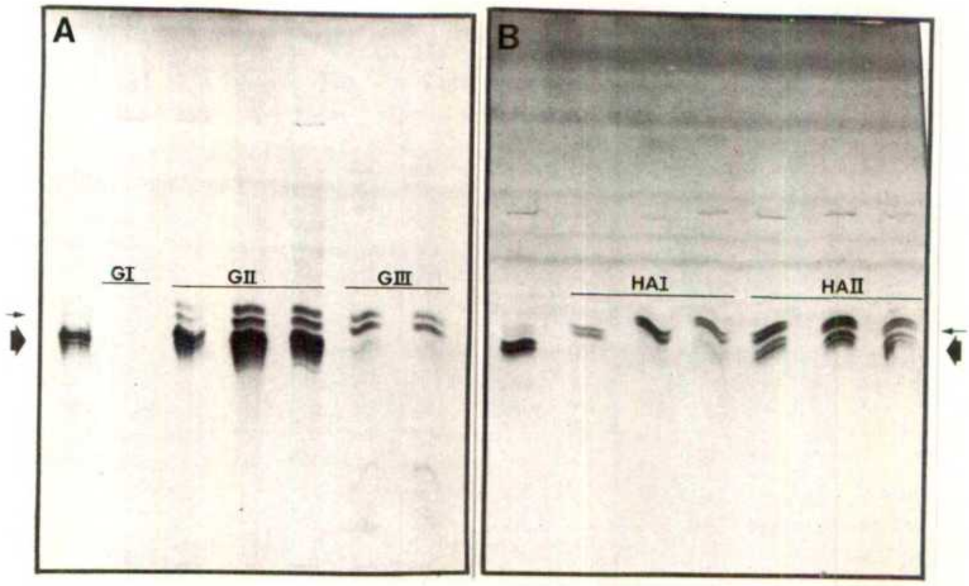


Fig 2. (A) Isoelectric focusing (IEF) zymograms of monocyte esterase fractions from gel-filtration chromatography on Sephadex G-200 (see Fig 1). ANA-Fast Blue BB. From left to right: unfractionated crude extract; peak GI; peak GII-leading edge, peak, trailing edge; peak GIII-peak, trailing edge. A characteristic closely spaced series of monocyte esterases is seen in PI region 5.8 to 6.3. Bold arrow indicates more anionic forms; fine arrow indicates less anionic forms. The amount of less anionic forms appears increased in fractionated material; these predominate in the GIII fractions. In this gel, only an expanded pH range of 5.0 to 8.0 was covered. The number of monocyte species is greater than that detected with standard gels covering pH range 3.5 to 8.0 (shown in right panel). (B) IEF zymograms of monocyte esterase fractions from hydroxyapatite (HA) adsorption chromatography (see Fig 3) pH 3.5 to 8.0. Conditions otherwise as described in left panel. From left to right: unfractionated crude extract; peak HAI-leading edge, peak, trailing edge; peak HAII-leading edge, peak, trailing edge. Again there is an increase in activity of forms of monocyte esterase with highest pI values as compared with crude extract; these predominate in peak HAI.

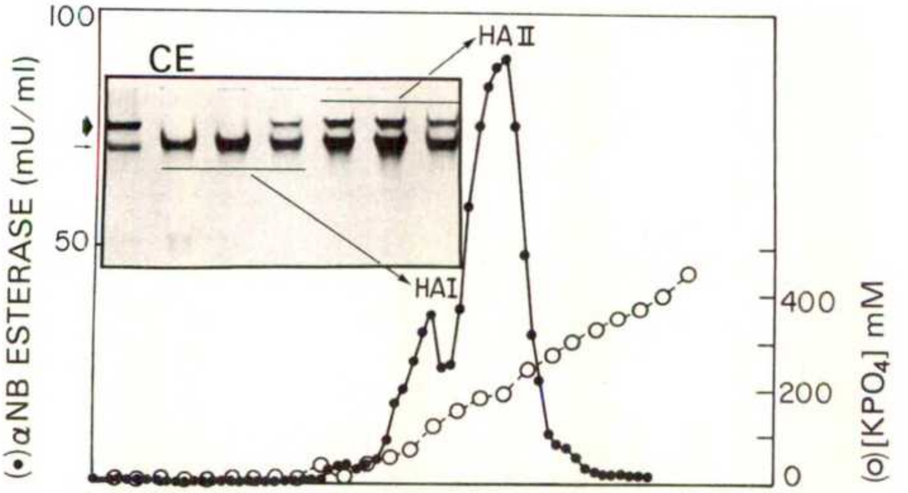


Fig 3. Analytical adsorption chromatography of ML-1 crude extract esterase. A 1-mL aliquot of crude extract containing 1,600 mU of ANB esterase in buffer C was chromatographed on a 1.5 × 6.0 cm hydroxyapatite (HA) column, and enzyme was eluted with a linear gradient to 500 mmol/L of KPO_4. Fractions of 1 mL were collected and assayed for ANB esterase. Inset: pH 9.5 polyacrylamide gel electrophoresis zymograms of monocyte esterase fractions from adsorption chromatography on HA. ANA-Fast Blue BB. From left to right: crude extract; HA peak I-leading edge, peak, trailing edge; HA peak II-leading edge, peak, trailing edge. Specimens were applied at standardized volume of 60 μL. Bold arrow indicates slower AB-2 form, fine arrow indicates the faster AB-1 form. Again the level of AB-1 activity appears increased in fractionated material as compared with crude extract, and AB-1 is the major form in peak HAI. The nonspecific acetyl esterases A1 and A2 are seen to elute just ahead of the HAI peak.

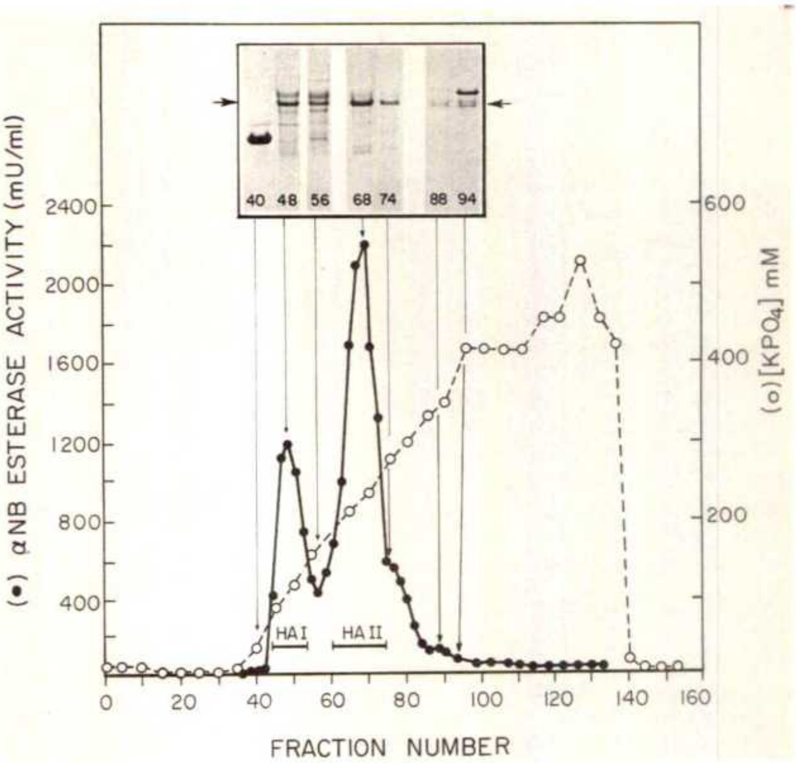


Fig 4. Hydroxyapatite-agarose adsorption chromatography of major esterase peak, GII, from gel-filtration chromatography. Experimental details are given in Materials and Methods section. Two peaks were eluted from the column: a minor peak, HAI, at ~100 mmol/L of KH_2PO_4, and a major peak, HAII, at ~200 mmol/L of KH_2PO_4. Inset: sodium dodecyl sulfate polyacrylamide gel electrophoresis of successive chromatography fractions. A standard volume of 300 μL from each fraction was analyzed for direct comparison of intensity of proteins. A single major protein, mol wt 62,000, is associated with both esterase peaks (arrows). The larger peak, HAII, is almost homogeneous with respect to this protein.

(Fig 5). To rule out contamination of the HAI fraction by the larger HAII peak, cuts of the HAI peak not including the trailing shoulder were examined and similar results were obtained. Conversely, the HAII peak consistently generated a minor HAI peak, even after the second rechromatography (Fig 6). Protein profiles closely matched enzyme peaks on rechromatography of the HAII fraction. The HAII peak showed the 62,000-mol wt subunit on SDS-PAGE in highly purified form (Fig 7). On pH 9.5 PAGE, the HAII peak enzyme showed a major protein that closely matched the R_F

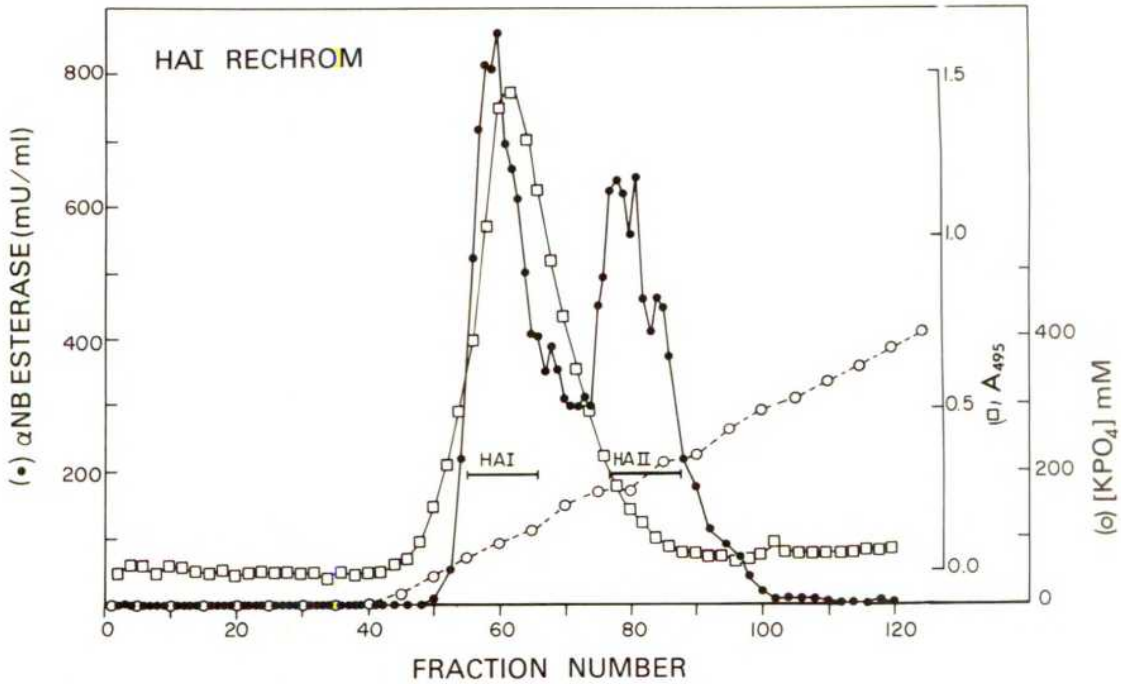


Fig 5. Rechromatography of hydroxyapatite (HA) peak HAI on HA. Experimental details are given in Materials and Methods section. A major HAII peak is generated from HAI. A Coomassie Blue protein peak is detected at 495 μm, which does not closely match either enzyme peak.

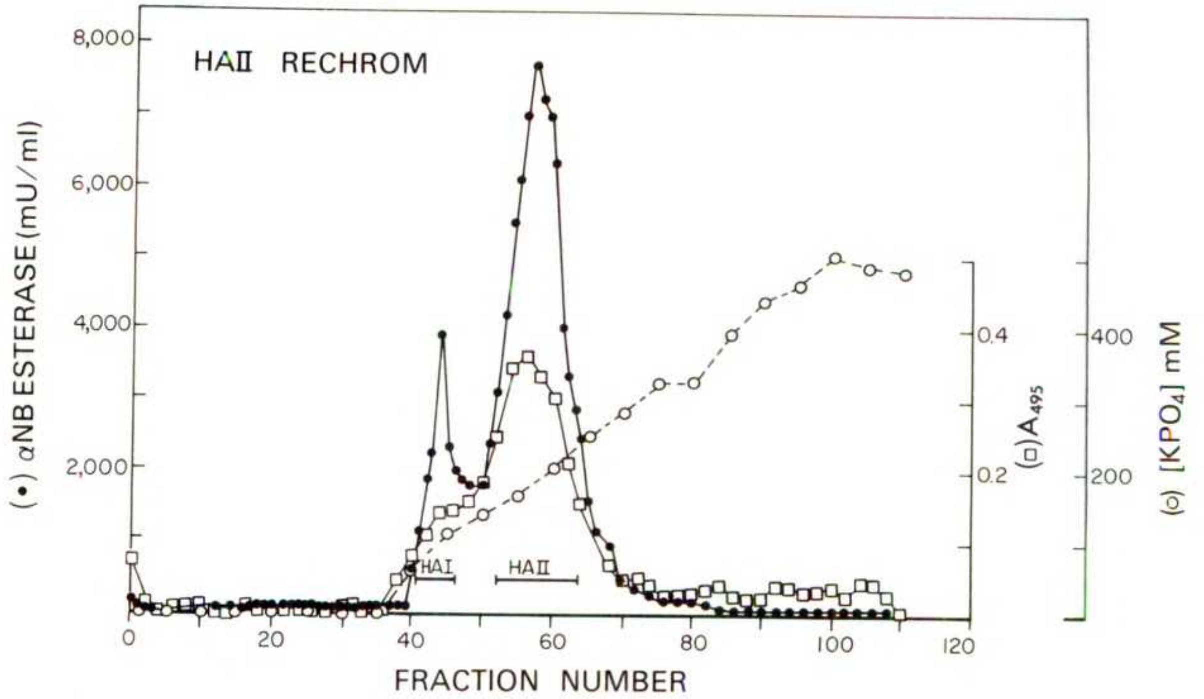


Fig 6. Rechromatography of hydroxyapatite (HA) peak HAII on HA. Experimental details are given in Materials and Methods section. A minor HAI peak is consistently generated from HAII. The Coomassie Blue protein peak at 495 μm matches the enzyme peaks closely.

of the AB-2 enzyme and a weaker series of two to three bands that matched the minor, AB-1 enzyme (Fig 8). It remains to be determined if the observed microheterogeneity in later stages of the purification represents hydrolytic processing of enzyme, enhancement of related minor forms, or greater resolving power of the system with purified material. The derivative minor HAI peak showed a similar but weaker pattern of bands. The rechromatographed HAI peak also generated similar esterase patterns on pH 9.5 PAGE; however, in this chromatography, protein stain showed residual contamination (HAI) or low levels of material (HAII) (Fig 8).

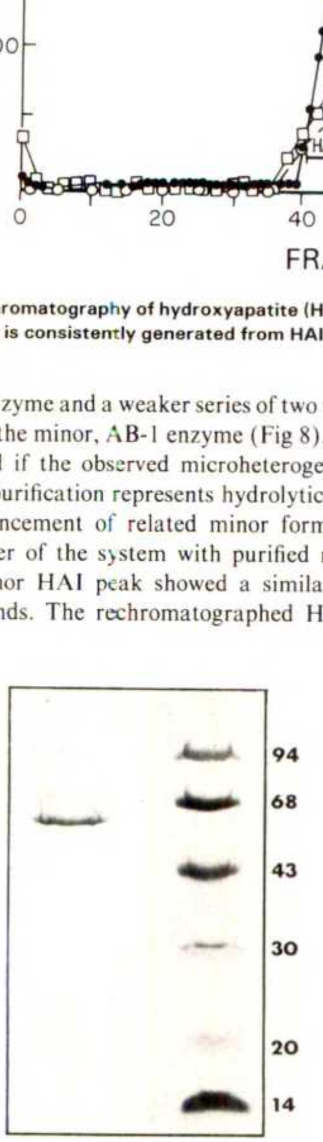


Fig 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of dissociated, dilapidated HAII peak enzyme. Left: the dissociated, delipidated enzyme generates a single major protein chain, mol wt 62,000. Right: mol wt standards include (top to bottom) phosphorylase B 94,000, bovine serum albumin 68,000, ovalbumin 43,000, carbonic anhydrase 30,000, soybean trypsin inhibitor 20,000, and lactalbumin 14,400.

On IEF, the HAI and HAII peaks from the first chromatography showed the entire series of esterase bands with HAI enriched for the less anionic forms and HAII enriched for the more anionic forms. This pattern approximated that of peaks obtained directly from chromatography of crude extract. When the HAI and HAII peaks were individually rechromatographed on hydroxyapatite, no apparent further segregation of forms with higher and lower PI values was apparent in the derivative HAI and HAII peaks obtained (Fig 9). The repurified HAII peak on IEF showed a close match of protein and enzyme bands (Fig 9).

Approximately 1,000 μg of protein representing 10% of original ANB esterase activity was recovered in the final HAII peak, representing a 350-fold purification based on activity *v* protein (Table 1). The calculated final yield was 140,000 mU of ANA esterase and 2,600 μg of protein based on rechromatography of the entire HAII fraction. Clearly, greater yields of highly purified HAII enzyme are obtainable after the first and second adsorption chromatography. The purification data suggest that monocyte nonspecific esterase comprises ~0.25% of starting protein in crude extract (26 mg of enzyme protein calculated in 10,300 mg of total protein).

The HAII peak enzyme was analyzed by gel-filtration chromatography on Sephadex G-200. Again, the characteristic monocyte elution pattern was seen, consisting of a major GII peak of a mol wt of ~205,000; a minor GIII peak of a mol wt of ~68,000; and a minor GI peak of high-mol-wt aggregates (Fig 10). On pH 9.5 PAGE, the repurified GII

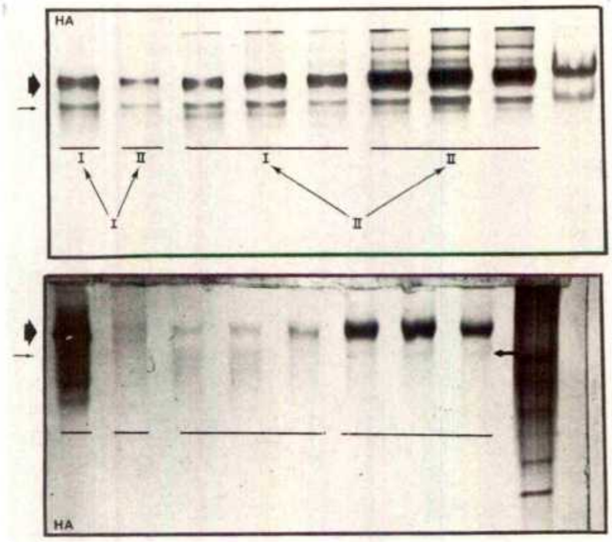


Fig 8. pH 9.5 polyacrylamide gel electrophoresis zymograms of fractions from hydroxyapatite (HA) rechromatography of peaks HAI and HAII. Top panel: ANA-Fast Blue BB. Bottom panel: Coomassie Blue protein stain. From left to right: HAI rechromatography peak HAI, peak HAII; HAII rechromatography peak HAI leading edge, peak, trailing edge; peak HAII leading edge, peak, trailing edge, crude extract control. All column fractions were applied at standardized volume of 10 μL for enzyme and 60 μL for protein. Bold arrow indicates AB-2 form; fine arrow indicates AB-1. These specimens were dialyzed and concentrated v 0.1 mmol/L of 2[*N*-morpholino]ethane sulfonic acid (MES) buffer, pH 6.4, containing 20% glycerol. Predialysis specimens of the same fractions in KPO$_4$ buffer showed predominance of the AB-1 forms in the HAI peaks. Here, the AB-2 form predominates in the same fractions. These results also indicate a marked tendency of AB-1 to generate AB-2 in these fractions, as seen on HA chromatography.

peak demonstrated the AB-2 form as a doublet, and the GIII peak demonstrated a mixture of AB-1 and AB-2 (Fig 10, inset). Possible reasons for the observed microheterogeneity are discussed above. On IEF, partial separation of more anionic (AB-2) forms in GII from the less anionic (AB-1) forms in GIII was again seen. The HAI peak showed a similar profile on G-200 rechromatography, except that the GIII peak was about twice the size of the GII peak (Fig 11). On pH 9.5 PAGE, this GII peak showed a mixture of AB-1 and AB-2, while GIII showed predominantly AB-1 (Fig 11, inset). IEF again demonstrated partial separation of less anionic (AB-1) forms in GIII from the more anionic (AB-2) forms in GII. These results suggest that hydroxyapatite adsorption chromatography separates monomers (HAI) and trimers (HAII) but that an association–dissociation equilibrium characteristic of each fraction is reestablished. This equilibrium is shifted toward the trimer in the purified HAII fraction and toward the monomer in the purified HAI fraction.

The GIII peak from preparative gel-filtration chromatography was of low specific activity due to heavy contamination by other proteins. On pH 9.5 PAGE and IEF, this peak showed the expected predominance of less anionic AB-1 forms. It also contained significant levels of the more anionic AB-2 forms.

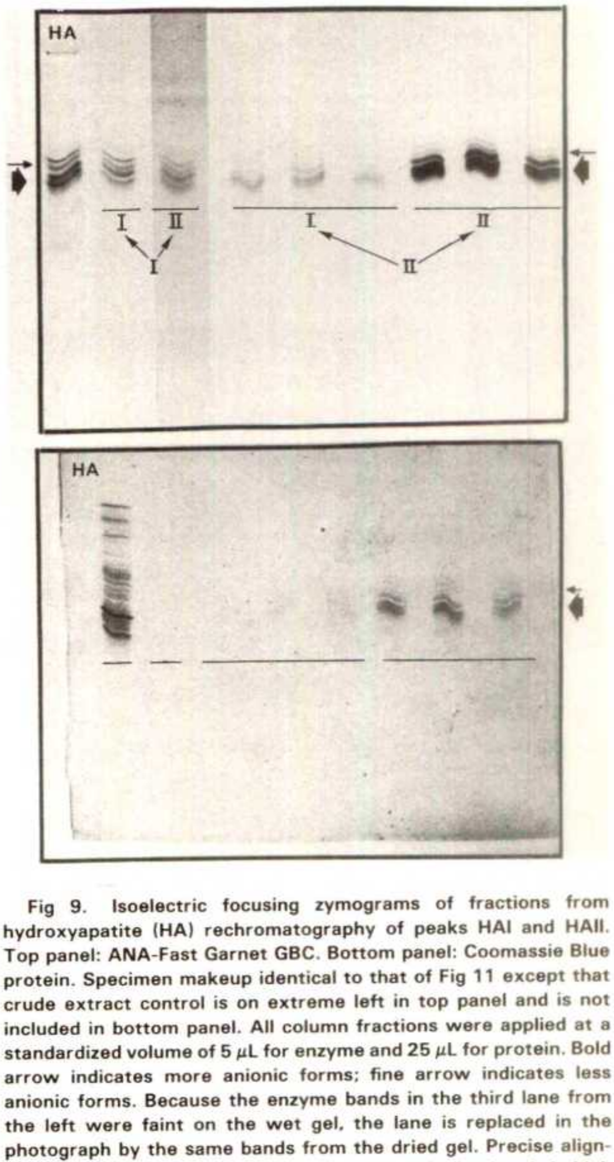


Fig 9. Isoelectric focusing zymograms of fractions from hydroxyapatite (HA) rechromatography of peaks HAI and HAII. Top panel: ANA-Fast Garnet GBC. Bottom panel: Coomassie Blue protein. Specimen makeup identical to that of Fig 11 except that crude extract control is on extreme left in top panel and is not included in bottom panel. All column fractions were applied at a standardized volume of 5 μL for enzyme and 25 μL for protein. Bold arrow indicates more anionic forms; fine arrow indicates less anionic forms. Because the enzyme bands in the third lane from the left were faint on the wet gel, the lane is replaced in the photograph by the same bands from the dried gel. Precise alignment of panels can be seen by horizontal ampholine bands in high pI range, which bind coupler dye nonspecifically and weakly.

Table 1. Purification of Monocyte Nonspecific Esterase

Fraction	Vol (mL)	ANB Esterase Activity (mU/mL)	Protein (mg/mL)	Specific Activity (mU/mg)	Total Activity	Recovery (%)
Crude extract	830	1,690	12.4	136	1,400,000	100
DH52 peak I	400	2,750	1.4	1,960	1,100,000	79
G200 peak II	130	4,100	0.53	7,740	530,000	38
HA-1 peak II	160	2,500	0.075	33,000	400,000	29
HA-2 peak II	22	10,000	ND	—	220,000	16
HA-3 peak II	10	5,000	0.104	48,000	50,000	10*

ANA, α-naphthyl acetate; ANB, α-naphthyl butyrate.

*An aliquot of HA-2 peak II containing 83,000 mU of ANB esterase was rechromatographed. Final yield is corrected accordingly. ANA esterase activity was 29% of ANB esterase activity in the crude extract. The ratio of ANA to ANB esterase decreased to 18% after DH52 chromatography and remained at 18% ± 1% through the final HAII fractions.

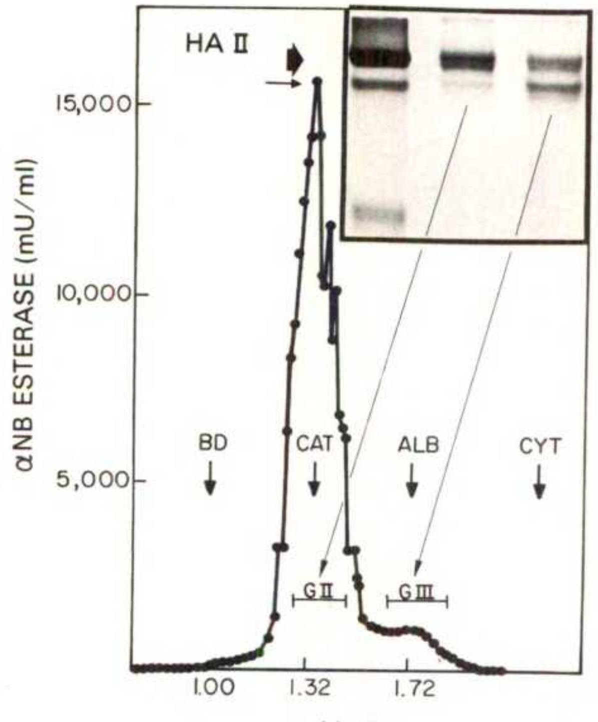


Fig 10. Analytical gel-filtration chromatography of hydroxyapatite (HA) peak HAII on Sephadex G-200. Conditions were similar to those described in Fig 1. Approximately 2 × 10⁵ mU of ANB esterase were applied. Inset: pH 9.5 polyacrylamide gel electrophoresis zymograms of Sephadex G-200 peaks, ANA-Fast Blue BB. From left to right, crude extract, peak GII, peak GIII; 2 mU of ANB esterase was applied from each G-200 peak.

Several enzymological parameters of the HAII peak enzyme were determined and found to be similar in all respects to those of normal monocyte enzyme.[20] These parameters include the broad neutral pH optimum for ANB and ANA esterase from pH 6.5 to 8.5, high sensitivity to DFP inhibitor (50% inhibition of ANB esterase at 1×10^{-7} mol/L, of ANA esterase at 2×10^{-7} mol/L of DFP), and high sensitivity to NaF inhibitor (50% inhibition of ANB esterase and of ANA esterase at 1 mmol/L of NaF).

DISCUSSION

Monocyte nonspecific esterase has been purified from cultured cells of AML-M4 cell line ML-1. The detergent-extracted membrane protein exists in the native state as a monomer of a mol wt of ~68,000 and a trimer of a mol wt of ~205,000 in a complex association–dissociation equilibrium. The trimer accounts for the bulk of extractable enzyme activity whereas the monomer is a relatively minor fraction. When dissociated and delipidated, the enzyme is seen to consist of a single major protein chain with a mol wt slightly lower than that of the native monomer—62,000. On pH 9.5 PAGE, the trimer migrates as the slower AB-2 form, and the monomer migrates as the faster AB-1 form. Analysis of crude extract shows that the trimer is associated with the more anionic forms, and the monomer is associated with the less anionic forms, as seen on IEF. A complex association–dissociation equilibrium and charge pattern of monomer and trimer is revealed with purified fractions. Further information is necessary to explain completely the various thermodynamic states of the enzyme in various environments and the details of the apparent noncovalent binding reaction of the subunits. A model incorporating the general features of monocyte nonspecific esterase subunit structure based on the data obtained from different systems is given in Fig 12.

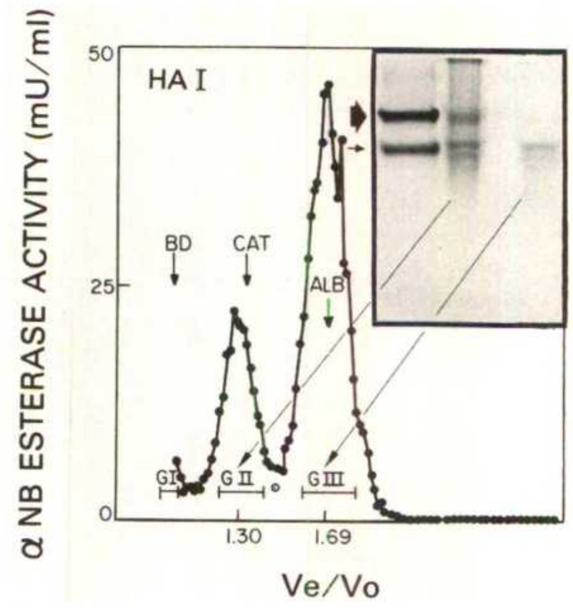
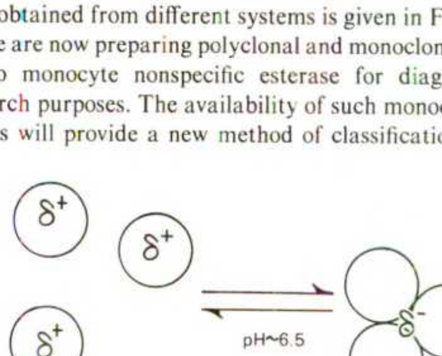


Fig 11. Analytical G-200 gel-filtration chromatography of hydroxyapatite peak HAI. Conditions were similar to those described in Fig 1. Approximately 1,000 mU of ANB esterase were applied. Inset: pH 9.5 polyacrylamide gel electrophoresis zymograms of Sephadex G-200 peaks, ANA-Fast Blue BB. Left to right: crude extract, peak GII, peak GIII. G200 peak specimens were applied at maximum loading (~0.4 to 0.5 mU of ANB esterase).

We are now preparing polyclonal and monoclonal antibodies to monocyte nonspecific esterase for diagnostic and research purposes. The availability of such monoclonal antibodies will provide a new method of classification of acute



MONOMERS		TRIMER
3 x 68,000	MW	1 x 204,000
pI 6.2-6.3	IEF	pI 5.8-6.1
AB-1	pH 9.5 PAGE	AB-2
peak GIII	G200	peak GII
peak HAI	HA	peak HAII

Fig 12. Model of monocyte nonspecific esterase subunit structure and noncovalent association–dissociation reaction-based chromatogram and zymogram data.

leukemias and for subclassification of acute myeloid leukemias based on nonspecific esterase. In addition, antibodies to the monocyte esterase will be of use in studies of myeloid/monocyte differentiation. We have recently presented evidence for activation of a myeloid-associated zymogen of monocyte esterase during monocyte/macrophage diffferentiation of HL-60 induced by phorbol esters.[16] An antibody probe for a zymogen epitope would furnish a new panmyeloid marker and allow analysis of the mechanism of zymogen activation. An antibody probe for an epitope present on active enzyme only will allow more precise and facile subclassification of acute myeloid leukemias into monocytic and nonmonocytic types.

Leukocytes contain a large array of nonspecific esterases that are detected with ANA, ANB, or similar substrates. Although these esterases share the common property of activity *v* naphthol esters, their diverse natural substrates and physiological roles remain unknown. Monocyte esterase is a membrane enzyme with the properties of a neutral serine carboxyl esterase. Neutral serine proteases, such as those represented by several digestive enzymes and blood-clotting enzymes, are the best known examples of this group of esterases.[4] Recently, a monocyte serine protease ectoenzyme with activity *v* serum amyloid A precursor was demonstrated.[7] A lymphocyte serine protease ectoenzyme of possible significance in blast transformation has previously been described.[21] Other types of carboxyl esterase may also operate by a similar reaction mechanism, among them lipoprotein lipase,[22] phospholipases A1 and A2,[23] and hormone-sensitive lipase.[24] The availability of purified monocyte esterase will allow us to screen protein, lipid, and other substrates definitively in an attempt to define the natural substrate and the physiological role of this ectoenzyme. The monocyte esterase must subserve some unique and important function in inflammation, metabolic processing, or growth regulation.[25] We are now in a position to study these interesting questions with the purified enzyme.

ACKNOWLEDGMENT

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Determination of Platelet Antigens and Glycoproteins in the Human Fetus

By Yves Gruel, Bernadette Boizard, Fernand Daffos, François Forestier, Jacques Caen, and Jean Luc Wautier

The autosomal recessive transmission of Glanzmann's thrombasthenia (GT) and Bernard-Soulier syndrome (BSS), together with requests of families who already had children with these diseases, prompted us to investigate the feasibility of their antenatal diagnosis. The preliminary step leading to the early detection of GT or BSS was to characterize, in the normal human fetus, the platelet antigens and glycoproteins (GPs) and to define their normal amounts on the membrane surface. Blood samples from 32 fetuses between 18 to 26 weeks of gestation were collected by direct puncture of the umbilical vein using an ultrasound-guided needle. Polyclonal antibodies from human origin directed against PL^{A1}, Lek^a antigens, and the GPIIb IIIa complex (IgGL), or murine monoclonal antibodies specific for GPIb (AN51, 6D1), GPIIIa (AP-3), or GPIIb IIIa (AP-2) were studied using platelet suspension immunofluorescence tests. The binding of each antibody was quantified using a cytofluorograph (Ortho 50H). PL^{A1} and Lek^a antigens were expressed in normal amounts on fetal platelets as early as 16 weeks of intrauterine life. The GPIIb IIIa complex quantified by polyclonal or monoclonal antibodies was in the same range in fetuses (IgGL = 427 ± 23 AUF, AP-2 = 459.5 ± 8.5; AP-3 = 536 ± 14) and in adults (IgGL = 420 ± 30; AP-2 = 498 ± 11; AP-3 = 515 ± 13). The platelet binding of antibodies that recognized GPIb was higher in fetuses (AN51 = 491.5 ± 14; 6D1 = 479 ± 15) than in adults (AN51 = 426.5 ± 9; 6D1 = 449 ± 8.7). These results suggest that immunological techniques can be applied as early as 18 weeks of gestation for the antenatal diagnosis of GT and BSS.

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GLANZMANN'S thrombasthenia (GT) and Bernard-Soulier syndrome (BSS) are hemorrhagic disorders caused by functional platelet abnormalities. These disorders have an autosomal genetic transmission and are characterized by a long bleeding time and defective platelet function such as aggregation or adhesion.[1,2] Recently, it was demonstrated that in GT, the defect in platelet aggregation induced by different agonists such as ADP, thrombin, or collagen was related to a defect of the platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa) complex.[1-3] The clinical severity and the frequency of the bleeding episodes varied among patients and, in several cases, required iterative platelet transfusions, which may lead to the development of antibodies directed against platelet antigens and refractoriness to platelet transfusion. In such conditions, bone marrow transplantation has been proposed as a possible treatment for severe GT.[4] The autosomal recessive transmission of GT and BSS, together with requests by families who already had children with this disease, prompted us to investigate the feasibility of antenatal diagnosis. The possibility of sampling fetal blood in the second trimester of pregnancy and the development of microtechniques using polyclonal and monoclonal antiplatelet antibodies enabled us to study fetal platelet GPs and PL^{A1} and Lek^a antigens, both of which are missing in GT.[5-10] This study is a necessary preliminary step leading to antenatal diagnosis of GT or BSS.

MATERIALS AND METHODS

Subjects

Blood was obtained from 17 normal volunteers and 12 pregnant women, mothers of the fetuses that were studied. Blood sampling was performed after informed consent in accordance with the provisions of Helsinki.

Fetal blood was obtained in 32 fetuses between 18 and 26 weeks of gestation by direct puncture of the umbilical cord with a needle guided by ultrasound.[6] In all cases, the blood sampling was done for antenatal diagnosis of either congenital toxoplasmosis, fetal rubella, or hemophilia. The blood not used for the diagnosis of the previously noted diseases was kept for platelet studies, and its volume varied between 0.5 and 1 mL. For each fetal sample, the absence of contamination by maternal blood was assessed by a Kleihauer Betke test,[7] and isoelectrofocusing of hemoglobin.[8] Cell size histograms of white and red blood cells estimated by Coulter "S plus II" were automatically recorded and compared to those from maternal blood. Fetal platelet count and volume were also systematically obtained (Coulter S plus II).

Antibodies

Three polyclonal antibodies from human origin were used: Anti-PL^{A1}, anti-Lek^a, and IgGL. The anti-PL^{A1} serum was obtained from the mother of a child with neonatal alloimmune thrombocytopenia. Its specificity was assessed by the study of platelets from PL^{A1}-positive and PL^{A1}-negative subjects and by immunoblot procedure.[9] The anti-Lek^a serum, which was obtained from a patient with a posttransfusion purpura, permitted a definition of a new platelet-specific alloantigen, Lek^a,[10] which is carried by membrane GPIIb, as demonstrated by immunoblotting.[9] IgGL was isolated from the serum of a polytransfused patient with GT.[11] IgGL reacted predominantly with GPIIb/IIIa complex, as demonstrated by immunoprecipitation.[12] Platelet GPs were characterized using four monoclonal antibodies: AP-2 (supplied by T. Kunicki) is a murine monoclonal antibody that reacts specifically with the complex formed by human platelet membrane GPIIb and GPIIIa but not with the individual GPs.[13] AP-3 (supplied by P. Newman), as recently demonstrated by indirect immunoprecipitation, reacts solely with GPIIIa.[14] AN51 (supplied by G. Tobelem) is a monoclonal antibody directed against the glycocalicin region of GPIb[15,16]; 6D1 (supplied by B. Coller) is also directed against the glycocalicin part of GPIb, but its specificity is probably different since it inhibits the binding of von Willebrand factor to platelets by 100% whereas inhibition obtained with AN51 is only 40% to 50%.[17]

Because the volume of fetal blood available for our study was small (often <500 μL), only 10 to 13 paired samples fetus/adult could be tested with each antibody.

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Indirect Platelet Immunofluorescence Tests

Platelet immunofluorescence tests were done according to a technique previously described.[18] In brief, platelets were prepared by differential centrifugation, washed three times in EDTA phosphate buffer, pH 7.4 (0.14 moL/L of Nacl, 0.0264 moL/L of Na2 HPO4–2H2O, 0.009 moL/L of EDTA-PBS), and then fixed with 1% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). PFA-fixed platelets (0.5 to 1.10^9/L in EDTA–PBS) were incubated with each antibody or antiserum for 30 minutes at 37 °C. Monoclonal antibodies AP-2, AP-3, AN51, and 6D1 were tested under saturating conditions, using a dilution which yielded the highest intensity of fluorescence, after binding to normal platelets. After incubation, platelets were washed three times in EDTA–PBS and the binding of each antibody was revealed by a second specific antibody conjugated with fluorescein. The platelet suspension was then examined with a Zeiss vertical fluorescence microscope (Zeiss, Wetzlau, FRG), and the antibody binding was quantified using a cytofluorograph (Ortho 50H, Boulogne, France).

Flow Cytometry Analysis

Platelet-associated fluorescence was quantified with a cytofluorograph Ortho 50 H linked to a computerized system (computer 2150, soft Ortho "threepitdif"). The cytofluorograph was equiped with an Argon-Ion laser 16405 "Spectraphysic" (500 mw, 488 nm). Each sample was introduced in a fluid capillary at a speed of 3,000 to 5,000 platelets per second, and three parameters were obtained: right-angle scatter, forward-angle scatter, and green fluorescence measurement. Integrated fluorescence signals were amplified using a logarithmic amplifier and were then digitized and displayed on a 1,000-channel scale. Results were expressed as arbitrary units of fluorescence (AUF). For each sample, mean fluorescence intensity was obtained by dividing the total fluorescence recorded by the number of platelets analyzed (10^4 platelets).

Statistical Analysis

Student's *t* test was used to compare the mean values of fluorescence, and Wilcoxon test was used to analyze the results obtained for paired samples, fetus/adult or fetus/mother.

RESULTS

Fetal Platelet Parameters

The mean platelet count in the 32 fetuses was 246 ± 54.10^9/L (mean ± SD), which is in the normal range for adults (150 to 400.10^9/L). The fetal platelet volume, as estimated by Coulter S plus II, was 9.69 ± 0.8 μ^3, greater than that obtained in normal adults (8.4 ± 0.9 μ^3). However, the difference was not statistically significant and could be due to the presence of large platelets which were observed in fetuses but not in adults.

Fetal Platelet Antigens

Platelet antigen PL^{A1} was present in all the fetal blood samples studied (N = 22). The apparent frequency of PL^{A1} antigen was the same in fetuses between 18 and 26 weeks of gestation (100%) and in adults (98%).[22] The quantity of PL^{A1} antigen on platelets, as estimated by indirect immunofluorescence and cytofluorometry, was similar in fetuses (433 ± 30 AUF, mean ± SEM) and in adults (427 ± 13) (Fig 1).

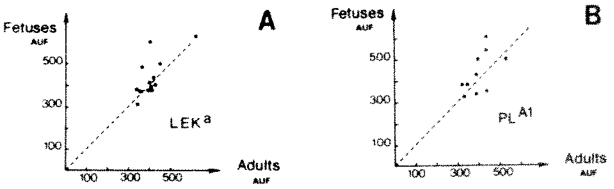

Fig 1. Fetal platelet antigens. Each point represents the mean fluorescence intensities (AUF) recorded for a pair (fetus/adult) studied during the same set of experiments. The platelet binding of anti-Lek^a (A) or anti-PL^{A1} (B) antibodies was quantified by cytofluorography. Points located on the dotted line correspond to a fetus/adult fluorescence intensities ratio of 1.

In this study, the heterozygous subjects for PL^{A1} antigen were not distinguished from homozygous individuals, since the relatives of the subjects were not tested.

The presence of Lek^a antigen was demonstrated on platelets in 23 fetuses between 18 and 26 weeks. Six fetuses were found to be Lek^a negative. Therefore, the apparent frequency of Lek^a (+) in fetal platelets (79.4%) is lower than that observed in adults (93.26%, N = 370), but the difference was not statistically significant. When Lek^a was expressed on platelets, the mean fluorescence intensity measured by cytofluorometry in fetuses (437 ± 25 AUF) was similar to that recorded with adult platelets (459 ± 15 AUF, N = 23) (Table 1, Fig 2). Two of the Lek^a-negative fetuses could be studied after their birth at the age of 6 months; they were still Lek^a negative.

Fetal Platelet Glycoproteins

GPIIb/IIIa: GPIIb/IIIa complex and GPIIIa were studied on platelets using three different antibodies: IgGL, AP-2, and AP-3. (Table 1, Figs 3 and 4).

IgGL. IgGL, which is specific for GPIIb/IIIa complex, reacted with all the fetal platelet samples studied (N = 23). The binding of IgGL to fetal platelets quantified in 11 cases was identical (427 ± 23 AUF) to that obtained with adult platelets (420 ± 30 AUF).

AP-2. AP-2 recognizes an epitope located on the GPIIb/IIIa complex and was found to react with the 16 fetal samples tested. The mean fluorescence intensity recorded in 12 cases appeared to be lower for the fetal platelets (459 ±

Table 1. Mean Fluorescence Values (± SEM)

	Immunofluorescence Intensity (AUF)	
Platelets	Fetus (Mean ± SD)	Adults (Mean ± SD)
Antigens		
PL^{A1}	433.0 ± 30.0	427 ± 13.5
LeK^a	441.5 ± 25.0	459 ± 15.0
Glycoproteins		
GPIIb IIIa, IgG	427.0 ± 23.0	420.0 ± 30.0
GPIIb IIIa, AP-2	459.5 ± 8.5	498.0 ± 11.0
GPIIIa, AP-3	536.0 ± 14.0	515.0 ± 13.0
GPIb, AN51	491.5 ± 14.0	426.5 ± 9.0
GPIb, 6D1	479.0 ± 15.0	443.0 ± 8.7

Values measured with the specific antibodies directed against platelet antigens PL^{A1}, Lek^a and platelet glycoproteins (Gp) IIb IIIa, Ib, in 10 to 13 fetuses and 23 adults. Only three fetuses were tested with 6D1.

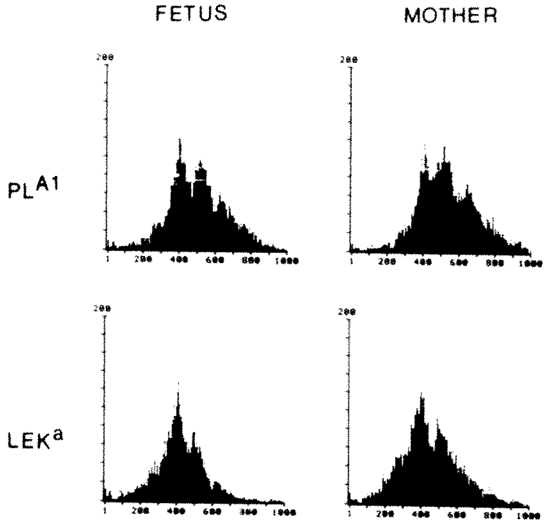

Fig 2. Fetal platelet antigens. Histograms of fluorescence recorded after binding of anti-PL^{A1} or anti-Lek^{a} antibodies to the platelets of a fetus and his mother. Fluorescence signals were amplified using a logarithmic amplifier which was then digitized and displayed on a 1,000-channel scale.

8.5 AUF) as compared with that obtained with adult platelets (498 ± 11 AUF, N = 23, $P < .02$, Student's t test). However, when the values of fluorescence recorded in the same experiment for paired samples fetus/adult or fetus/mother were analyzed by Wilcoxon test, the difference did not remain statistically significant.

AP-3. Fetal platelet binding of AP-3, specific for a GPIIIa epitope, was demonstrated in the 22 fetuses studied with this antibody. At variance with that obtained with AP-2, the fluorescence intensities with AP-3 were identical in fetal and adult populations: fetuses = 523 ± 18.5 AUF, N = 12; adults = 515 ± 15 AUF, N = 23.

GPIb. (Table 1, Figs 3 through 5) GPIb was studied on human fetal platelets using two monoclonal antibodies, AN51 and 6D1, which are both directed against determinants located on glycocalicin. The binding of AN51 was demonstrated in all fetuses studied, and the mean fluorescence intensity was higher (492 ± 13:5 AUF, N = 12) than in adults (426 ± 9 AUF, N = 23, $P < .01$). The platelet binding of 6D1 was also higher for the three fetuses tested (479 ± 15 AUF) as compared with that of adults (449 ± 8.7 AUF). In an attempt to standardize the results, we have calculated the ratio of the values of fluorescence intensity

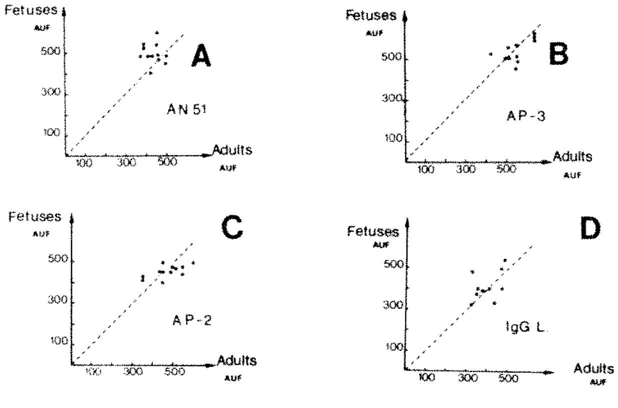

Fig 3. Fetal platelet glycoproteins. Each point represents the mean fluorescence intensities (AUF) recorded for a pair (fetus/adult) after platelet binding of (A) AN51 (GPIb), (C) AP-2 (GPIIb/IIIa), (B) AP-3 (GPIIIa), and (D) IgGL (GPIIb/IIIa). GP, glycoprotein.

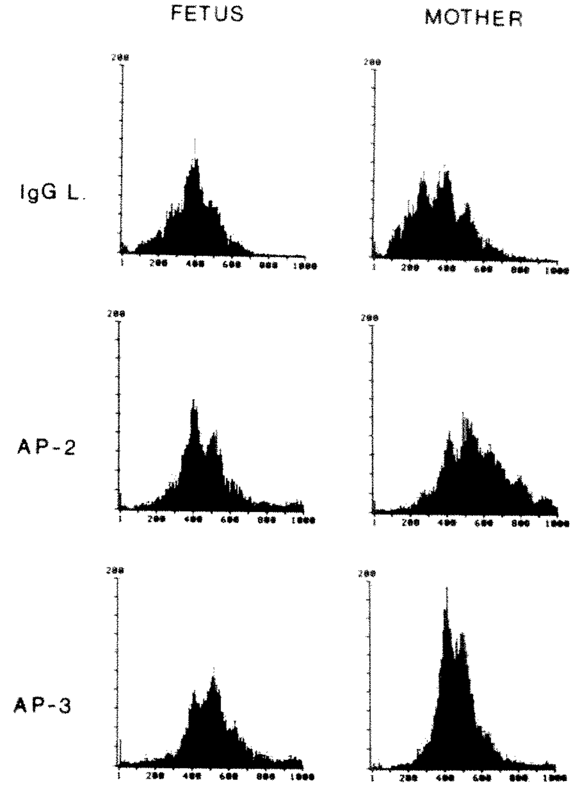

Fig 4. Histograms of fluorescence recorded after binding of IgGL (GPIIb/IIIa), AP-2 (GPIIb/IIIa), and AP-3 (GPIIIa) to platelets. GP, glycoprotein.

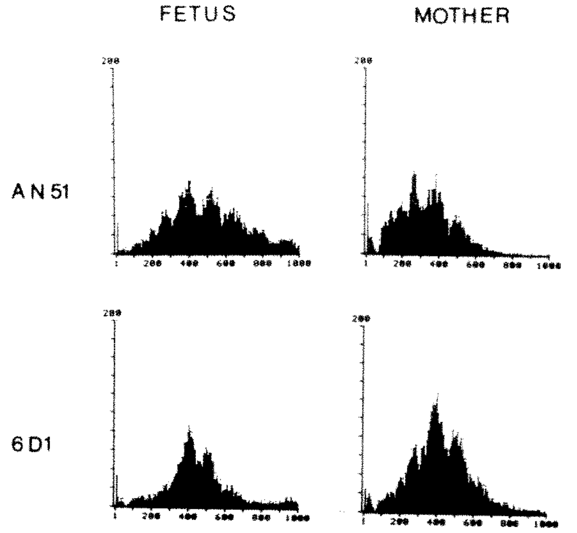

Fig 5. Histograms of fluorescence recorded after binding of AN51 and 6D1 (GPIb) on the platelets of a fetus and his mother. GP, glycoprotein.

obtained with a monoclonal antibody directed against GPIb and one against GPIIb IIIa or GPIIIa, namely AN51/AP2 and AN51/AP3. The ratio of AN51 to AP2 in fetuses was higher than in adults; 1.108 ± 0.062 and 0.869 ± 0.0029, respectively ($P < .01$). The ratio of AN51 to AP3 was also higher in fetuses (1.013 ± 0.058) than in adults (0.838 ± 0.020). The difference was statistically significant ($P < .01$) and demonstrated a relative increase in GPIb reactivity with AN51 in the human fetus.

DISCUSSION

The platelet counts of the 32 fetuses that were sampled in our study were similar to those of adults. This result is in agreement with previous reports, which found no significant variation of the fetal platelet number from 18 weeks to the end of pregnancy.[19] As estimated by Coulter S plus II, the mean platelet volume appeared slightly greater in fetuses as compared with that in normal adults. Bleyer et al reported that circulating platelets existed in 11-week-old fetuses, and some cells, resembling mature platelets, were slightly larger and contained fewer granules.[20] In contrast, Holmberg et al found no significant difference between fetal and adult mean platelet volume and used the platelet volume as a criterion to exclude the diagnosis of Wiskott-Aldrich syndrome in an 18-week-old fetus.[21]

We have studied fetal platelet antigens and GPs using an indirect immunofluorescence technique associated with flow cytometry, since this method can be applied to the low blood volumes (1 to 2 mL) that can be obtained from human fetuses during the second trimester of pregnancy.

Our results showed that PL^{A1} and Lek^a antigens are expressed on fetal platelets as early as 16 weeks of gestational age. The frequency of PL^{A1} antigen in the group of fetuses was similar to that obtained in adults.

After the binding of anti PL^{A1}, cytometry analysis showed identical values in fetal and adult groups. The expression of PL^{A1} antigen in 16-week-old fetuses is complete and may explain the high frequency of immunization in PL^{A1}-negative women during their first pregnancy, estimated at 59%.[22] The early expression of PL^{A1} could play a role in the early occurrence of the fetal thrombocytopenia due to anti-PL^{A1} antibodies that has been observed after 20 weeks of gestation.[23]

The expression of Lek^a does not seem to be different in fetuses and adults. Whatever the gestational age between 18 and 26 weeks, the amount of Lek^a antigen was similar. The study of membrane GPs showed that GPIIb/IIIa, as demonstrated by the binding of monoclonal or polyclonal antibody, appeared to be normally expressed on fetal platelets as early as 18 weeks of gestational age. The difference in the AP-2 binding was not significant, and the fluorescence intensities recorded with AP3 and IgGL were identical in adults and fetuses. In these conditions, the amount of GPIIb/IIIa complex appeared to be similar in fetuses and adults. However, AP-2 recognizes a conformational structure of the complex, and we cannot exclude a slight difference between fetuses and adults. When radiolabeled monoclonal antibodies were used in adults, the number of specific sites per platelet for AP-2 was 57,400 ± 9,700[13] and 40,200 for AP-3.[14] Our technique did not allow a precise estimation of the number of specific sites per platelet; however, because the results with AP-2 and AP-3 were similar in adults and in fetuses between 18 and 26 weeks of gestation, we can postulate that the number of GPIIb/IIIa is similar in the two groups. In addition, a strong correlation between fluorescence values obtained by cytofluorometry and the number of antigens sites expressed on cell surface has been reported.[24]

GPIb was characterized on all fetal platelet samples studied, and the binding of AN51 appeared significantly higher as compared with that in adults. Moreover, binding of 6D1, which may react with a different GPIb epitope, was higher in fetuses. The number of specific GPIb sites recognized by AN51 and 6D1 on adult platelets is similar: 26,000 to 29,000 per platelet.[17-25] The reactivity of GPIb was higher in fetal platelets. This did not seem to be due to the slightly increased platelet volume, since the individual fluorescence ratios—AN51/AP-2 or AN51/AP-3—were significantly higher in fetuses. The normal expression of GPIb and GPIIb/IIIa in fetuses indicates that antenatal diagnosis of congenital platelet disorders such as GT or BSS is feasible. In GT type I, GPIIb/IIIa complex is undetectable, whereas GPIIb/IIIa appears to be normally expressed on fetal platelets as early as 18 weeks of gestational age. As a result, antenatal diagnosis of GT could be performed at this term. In a similar manner, diagnosis appears to be possible for GT type II subjects who have ~15% of the normal level of GPIIb/IIIa. Recently, new patients with thrombasthenia variants presenting all the functional abnormalities observed in type I but a normal or subnormal level of membrane GPIIb/IIIa were described.[26,27] Therefore, immunological techniques cannot be applied to the diagnosis of these variants, emphasizing the importance of the family study using several different techniques as a prerequisite step in the approach of the antenatal diagnosis of GT. The antenatal diagnosis can only be done after a complete study of the family members by means of functional, immunological, and biochemical techniques associated with appropriate genetic information. The results obtained in this study indicate that the antenatal diagnosis is feasible at 18 weeks of gestation. This appears to be progress but is still unsatisfactory from an ethical point of view. We hope that the technology using DNA analysis that has been developed for hemoglobinopathies or hemophilia can be applied at an early stage of pregnancy to the detection of congenital thrombopathies.

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Treatment of Hairy Cell Leukemia With Recombinant α-Interferon

By Jorge R. Quesada, Evan M. Hersh, John Manning, James Reuben, Michael Keating,
Edward Schnipper, Loretta Itri, and Jordan U. Gutterman

Thirty patients with hairy cell leukemia were treated with recombinant interferon α-A (rIFNαA; Roferon-A); seven were previously untreated. Nine complete and 17 partial remissions were documented by bone marrow core biopsies. All patients' peripheral blood hematologic indexes either improved or normalized. Twelve of 13 patients with retroperitoneal or mediastinal adenopathy obtained remissions of tumor masses. All seven patients with splenomegaly showed prompt reduction in the size of the spleen to normal size. The incidence of complete remissions was significantly higher ($P = .02$) in previously untreated patients (5 of 7) than in those in whom splenectomy had been performed (4 of 23), a result presumed to be related to the pretreatment tumor burden. rIFNαA was well tolerated; mild fatigue was the most frequent complaint. In most patients, tumor remissions resulted in an improved quality of life: they eliminated the need for transfusing blood products and reduced the incidence of infections, and immune deficits were apparently restored in some of the patients. We conclude that rIFNαA is an effective therapy for all stages of hairy cell leukemia including previously untreated or newly diagnosed patients.

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WE HAVE previously shown that partially purified α-interferon (IFNα) consistently induces bone marrow remissions in patients with hairy cell leukemia (HCL), resulting in improved or normalized peripheral blood indexes.[1] Subsequent experience with IFNα has shown that patients with all clinical stages of HCL, including those with profound pancytopenia, active life-threatening infections, or large retroperitoneal lymph node involvement, responded well to daily administration of this agent.[2] The objective of the present study was to investigate whether a highly purified cloned IFNα had similar activity. Recombinant IFNα (rIFNαA) has been shown to induce remissions in other B-cell malignancies, including low-grade lymphomas and multiple myeloma.[3-5]

We present data indicating that IFNα is indeed primarily, if not solely, responsible for the antitumor activity observed in HCL. Furthermore, rIFNαA induced tumor remissions in patients who had not had splenectomy, perhaps indicating that this surgical procedure may not be necessary for these patients.

MATERIALS AND METHODS

The diagnosis of HCL was made on the basis of clinical characteristics and laboratory demonstration of typical hairy cells in peripheral blood, bone marrow, or tissue specimens; evidence included a positive tartrate-resistant acid phosphatase stain. The effects of treatment were assessed by standard hematologic methods. Bone marrow was obtained every 4 to 12 weeks from multiple sites in the iliac crests and was evaluated under light microscopy and by flow cytometry, using monoclonal antibodies to leukocyte surface antigens. A complete remission (CR) was defined as an absence of hairy cells from peripheral blood, bone marrow aspirate, and biopsy specimens on at least two consecutive occasions and restoration of the hemoglobin level to ≥ 12 g/dL, the absolute granulocyte count to $\geq 1{,}500/\mu L$, and the platelet count to $\geq 100{,}000/\mu L$. A partial remission (PR) was defined as a decrease of $\geq 50\%$ in the bone marrow leukemic infiltrate (percentage of cellularity times percentage of hairy cells) from pretreatment values and restoration of the peripheral blood values as indicated above. In patients who initially had splenomegaly or in whom computerized tomography (CT) of the abdomen revealed retroperitoneal adenopathy, remission criteria also included: CR, spleen size reduced to normal or disappearance of nodal masses; and PR, $\geq 50\%$ reduction in size of the spleen or nodal masses. Minor responses were defined as restoration of one or more abnormal hematologic indexes or as bone marrow remission, but without recovery of all hematologic indexes.

rIFNαA (Roferon-A) was provided by Hoffmann LaRoche (Nutley, NJ). The purified protein was homogenous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and had a specific activity of 2 to 4 × 10^8 U/mg of protein. rIFNαA was administered by either intramuscular or subcutaneous injection daily for the first 4 to 6 months and three times a week until completion of 1 year of treatment. The first 12 patients received an initial dose of 10 to 12 × 10^6 U which was subsequently reduced to 3 × 10^6 U due to the more pronounced side effects of the higher induction dose. The remaining patients received 3 × 10^6 U throughout the study. All patients signed informed consents in accordance with institutional policies.

Surface markers. Bone marrow and peripheral blood mononuclear cells were fractionated according to cell volume and granularity with an Ortho Spectrum III cell analyzer.[6] Cell-surface markers were determined using the fluorescein-labeled monoclonal antibodies OKT3, OKT4, OKT11, OKT8, OKM-1, and OKIa (Ortho Diagnostic Systems, Raritan, NJ) and the B-1 antigen (Coulter Electronics, Hialeah, Fla). Surface immunoglobulins were determined after incubation with $(Fab')_2$ fragments of polyclonal antibodies specific for heavy or light immunoglobulin chains of goat anti-human IgM and IgD (Kallested, Austin, Tex). Anti-T cell activation antigen (anti-Tac) antibodies were a gift from Dr Thomas Waldmann (National Cancer Institute, Bethesda, Md).

Delayed hypersensitivity tests. Delayed hypersensitivity to recall antigens was determined by using the multitest battery (Merieux Institute, Miami, Fla).[7] This battery consists of tetanus (5.5 × 10 tetanus U/mL), *Streptococcus* (2 × 10 *Streptococcus* U/mL), tuberculin (3 × 10 US tuberculin U/mL), *Candida* (2 × 10 *Candida* U/mL), trichophytin (150 trichophytin U/mL), *Proteus* (150 *Proteus* U/mL), and a glycerol control. Simultaneous

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application of the antigens and control was accomplished by a single-stroke inoculation using the Multitest-CMI device. The scoring and interpretation of the responses have been described previously.[8] Anergy was defined as reactivity to 0 or 1 antigen(s), hypoergy as reactivity against 2 or 3 antigens, and adequate reactivity as a positive reaction to >3 of 7 antigens.

Statistics. Chi-square and Mann-Whitney tests were used for analysis. All *P* values <.05 were considered statistically significant.

RESULTS

Twenty-five men and five women, aged 25 to 59 years (median: 48 years) were treated beginning in January 1984. Twenty-three patients had undergone splenectomy, and 11 of these (48%) had subsequently received chemotherapy or hormonal therapy prior to treatment with rIFNαA. In all 23 patients, either prior treatment had failed or there was evidence of progressive disease at the time the patient was started on treatment. The remaining seven patients had not received any prior treatment and had splenomegaly and various degrees of pancytopenia initially. Twenty of the 30 patients (67%) had required transfusions of RBCs and 23 (77%) had a history of infections or fever of undetermined origin. The phenotype of the leukemic cells in the bone marrow was obtained in 21 patients. Hairy cells were larger and more granular than normal marrow mononuclear cells and exhibited surface immunoglobulins, Ia antigen, or B1 antigen, indicating their B cell origin. Hairy cells expressed the Tac antigen as previously reported[9] in all patients, whereas the MO1 antigen was present in only 65% of them.

Nine complete and 17 partial remissions were observed (Table 1). The remaining four patients had shown hematologic improvement but have not achieved a remission status, either because of persistent granulocytopenia (two patients), thrombocytopenia (1 patient), or a lack of bone marrow remission (2 patients).

Figures 1 through 3 depict the pretreatment and posttreatment values over time for platelets, leukocytes, and hemoglobin, respectively. All patients, regardless of their remission status, benefited from treatment, as evidenced by the progressive rise in these hematologic indexes. The platelet counts of 21 of the 22 patients with thrombocytopenia normalized within 4 months (Fig 1). Platelet count was usually the first blood index to improve, often within 2 to 3 weeks of initiating treatment. No adverse effects were seen in patients with normal pretreatment platelet counts. Figure 2 illustrates the decline in the leukocyte count of seven patients who had leukocytosis. Figure 2 also illustrates a rapid clearance of circulating leukemic cells in 18 of the 20 patients who had hairy cells in the peripheral blood. No patient had circulating leukemic cells beyond 6 months of therapy. Concomitantly, we observed a steady upward trend in the granulocyte counts of all patients (Fig 2), and the absolute monocyte counts increased from a pretreatment mean value of 0.02×10^3 monocytes/μL to 0.16, 0.18, and 0.27×10^3 monocytes/μL at 4, 8, and 12 months, respectively.

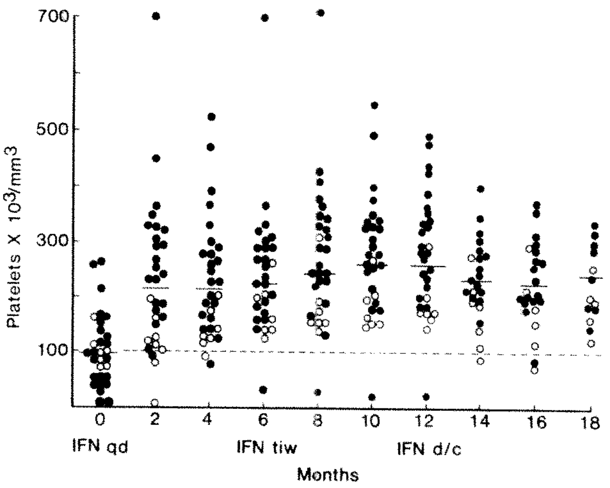

Fig 1. Platelet counts before and after treatment. Counts in previously untreated patients (open circles); mean platelet count (solid lines); value used in criteria of response (dashed line).

Table 1. rIFNαA in Hairy Cell Leukemia: Tumor Responses

Type of Response	Patients Responding (%)		Duration of Remission (mo) Median (Range)	
Complete remission	9	30	10+	6-20+
Partial remission	17	57	10+	4-22+
Minor responses	4	13	—	—

rIFNαA, recombinant interferon αA.

Figure 4 depicts the results of serial bone marrow core biopsies. rIFNαA consistently produced a steady reduction in the cellularity of the bone marrow and a decrease in the leukemic infiltrate. The time required to achieve a remission status in the bone marrow varied from 1 to 10 months (median: 3.5 months).

Thirteen previously treated patients presented with retroperitoneal adenopathy, identified by CT of the abdomen: eight of these patients had extensive nodal involvement, including four who also had mediastinal adenopathy. Size of the tumor masses decreased 50% to 100% within 2 to 6 months of therapy in all but one patient.

Remissions of previously untreated patients. Five of the 7 (71%) previously untreated patients achieved a CR in contrast to 4 of 23 (17%) previously treated ($P = .02$). A comparison of these two groups showed that the median time between diagnosis and onset of treatment with rIFNαA was 15 months (range 2 to 21 months) in the untreated patients and 23 months (range 9 to 121 months) in those previously treated ($P = .01$). The leukemic infiltrate was also significantly different ($P = .01$) among previously untreated patients (median: 10%; range 5% to 85%) and those previously treated (median: 55%; range 15% to 86%). All seven previously untreated patients had splenomegaly, and two also had hepatomegaly. The spleen and liver were clinically undetectable within the first 3 months of therapy. The median platelet count in untreated patients at 12 months from the onset of treatment (154,000/μL) was however significantly lower ($P = .005$) than the platelet counts of splenectomized patients (266,000/μL). No differences in the posttreatment hemoglobin values or granulocyte counts were found between these two groups of patients.

Duration of remissions. All patients completed 12 months of treatment, and in 25 rIFNαA was discontinued.

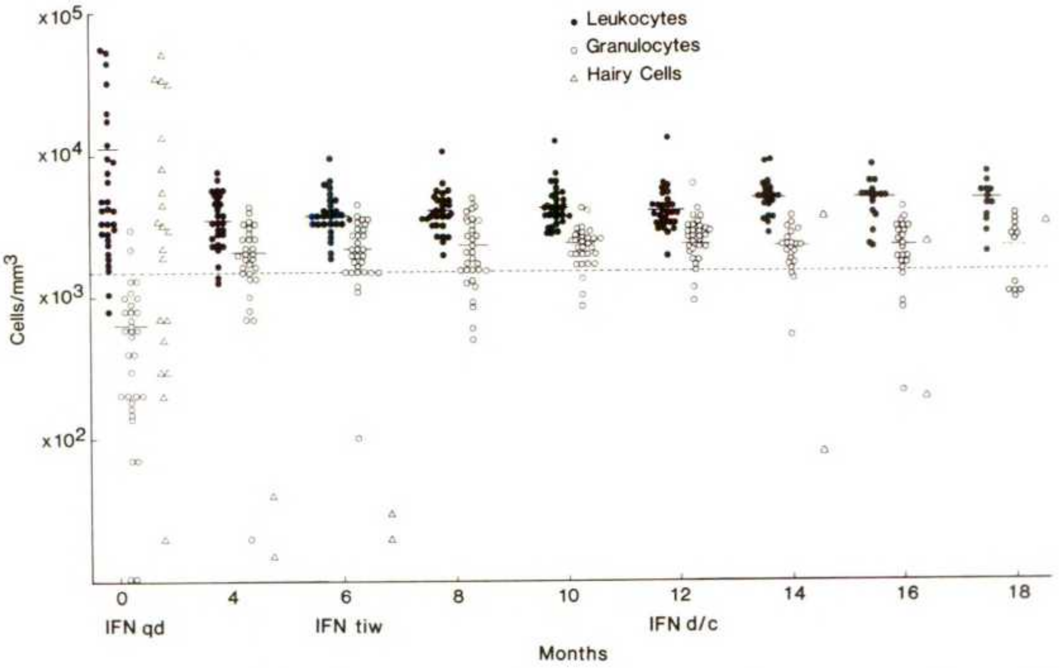

Fig 2. Semilogarithmic plot of the leukocyte, granulocyte, and hairy cell counts before and after treatment. Mean values (solid lines); value used in criteria of granulocytic response (dashed line). For clarity only, values >0 are shown for circulating hairy cells.

All patients are alive with a median follow-up >20 months (range 15 to 26 months). In eight of the 25 patients (33%) in whom treatment was discontinued, a progressive decline in the granulocyte count, increase in the bone marrow index, and reappearance of circulating hairy cells (two patients), were observed (Fig 2). This clinical relapse occurred within 3 to 10 months (median 6 months) after cessation of treatment in six patients who attained PRs, and one patient each with a CR and minor response. Reinitiation of treatment resulted in reinduction of remission in five of the eight patients who have completed 3 months of administration. Furthermore, in 11 other patients (42%) an increase in the bone marrow leukemic index has become evident, but has not warranted reinitiation of treatment because all hematologic indexes remain stable. In six patients (three CRs and three PRs) remission is sustained at 6 to 22+ months (median 12+ months) after discontinuation of rIFNαA.

Toxicity. In general, tolerance and compliance were excellent. Initial side effects consisting of transient fever and mild fatigue occurred in most patients. Symptoms were more intense in the first 12 patients who received the higher dose and therefore had the dose reduced. As treatment continued and hematologic indexes improved, most patients reported no or only mild adverse effects, mainly consisting of fatigue. However, all patients were able to continue or resume normal daily life activities, including strenuous exercise for some young patients.

Ten patients reported a decrease in libido during treatment. Three patients developed arthritis and/or vasculitis with mild inflammation of small joints of the hands while

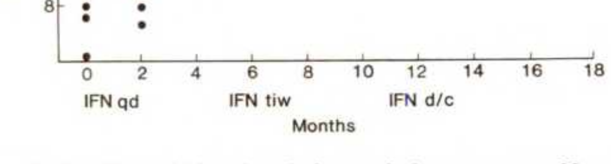

Fig 3. Hemoglobin values before and after treatment. Mean values (solid lines); value used in criteria of response (dashed line); previously untreated patients (open circles).

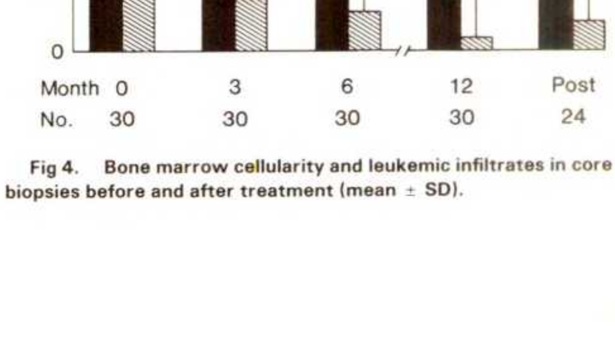

Fig 4. Bone marrow cellularity and leukemic infiltrates in core biopsies before and after treatment (mean ± SD).

receiving rIFNαA. These symptoms resolved spontaneously in two patients and required administration of corticosteroids in one. Screening for collagen disease-related antibodies or antigens was negative. A causal relationship with rIFNαA therapy is considered remote, because features of autoimmune disorders complicate the natural course of the disease in as much as 24% of the patients.[10]

Infections. Twenty-three patients had a history of previous infections or episodes of fever of unknown origin prior to treatment, ranging from one to four episodes (median: 2.5), but none of the patients had active clinical infection at the onset of treatment. Two of 12 (17%) patients who received the higher dose and 2 of 18 (11%) who received 3 × 10^6 U had major infections in the first 8 weeks of treatment (cryptococcal pneumonia, meningoccocemia, gram-negative bacilli pneumonia and soft tissue abscess, and *S viridans* endocarditis). In four additional patients from each group, the initial treatment course was complicated by minor upper respiratory, soft-tissue, or skin infections, or by fever of undetermined origin that merited antibiotic prescription. Among these, in three patients in the high-dose and four in the low-dose groups, circulating granulocytes decreased within the first 2 weeks of treatment. This decrease did not correlate with the onset of infection. No major or minor infections have been observed beyond the first 8 weeks of rIFNαA therapy in any of the patients.

Immunologic tests. Twenty-one patients underwent delayed hypersensitivity skin testing before and after 12 months of treatment. The number of positive reactions increased in 6 of 12 anergic and 3 of 5 hypoergic patients (Fig 5). In addition, 11 of 26 patients tested showed an inverted T helper/suppressor (T/S) ratio prior to treatment. In 5 of the 11 patients, a normal T/S ratio was restored at 6 or 12 months after beginning treatment with rIFNαA (data not shown).

DISCUSSION

This study confirms the efficacy of IFNα in treating hairy cell leukemia. Furthermore, it proves that the IFN molecule itself is almost certainly responsible for the antitumor effects originally described with a partially purified preparation of IFNα.[1,2] While we were preparing this report, other groups of investigators reported the activity of rIFNαA and lymphoblastoid IFN in HCL.[11-17] The scantiness of complete remissions in some of these studies[11-13] may be explained by their different treatment-induction schedule (three times a week), their shorter follow-up periods, or the more advanced stages of disease of their patients (none of these studies included untreated patients.[11-13]).

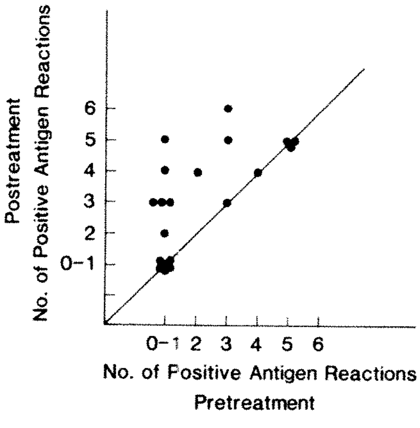

Fig 5. Comparative plot of pretreatment and posttreatment results of delayed hypersensitivity skin tests.

An important contribution of the current work is the result observed in previously untreated patients with splenomegaly. rIFNαA was effective in inducing tumor remission and reducing the size of the spleen, producing a significantly higher incidence of complete remissions than was achieved in previously splenectomized patients. The most likely explanation for this finding is the earlier stage of the disease of the untreated patients, as shown by a shorter interval between diagnosis and initiation of treatment and a lower level of bone marrow leukemic infiltrate. Other studies that have included untreated patients also report a larger number of complete remissions[16,17] similar to the results of this study.

The significant difference between the median platelet count after 12 months of treatment of previously untreated patients and of those who had been splenectomized suggests that the spleen may continue to consume platelets. Conceivably, some of these patients may benefit further from splenectomy. If so, the surgical risk should be decreased by their improved peripheral blood indexes resulting from rIFNαA therapy.

Discontinuation of rIFNαA after 12 months of administration resulted in clinical relapse of the disease in 33% of the patients within 3 to 10 months, suggesting the need for prolonged therapy beyond 1 year in some patients. In patients treated for 2 years with partially purified IFNα, only 3 of 21 (14%) have required reinitiation of treatment at 10, 12, and 14 months after discontinuation of therapy (unpublished observations, April 1986). Because reinduction of tumor response may be accomplished with reinitiation of treatment, periodic administration of rIFNαA may prove satisfactory for some of these patients.

rIFNαA demonstrated an excellent therapeutic index. Daily doses of 3 × 10^6 U were as effective as higher doses and better tolerated. Tumor remissions have resulted in an improved quality of life for our patients, including no further need for transfusion of blood products and an apparent reduction in the incidence of infections, the major morbidity factors associated with HCL. Furthermore, our preliminary data suggest that tumor response may also improve some of the immunologic deficits present in these patients. In this regard, we have also reported on the ability of rIFNαA to improve deficient humoral immunity in responsive patients with multiple myeloma.[5]

Caution must be exerted during the initial 2 months of treatment because of the possibility of complicating infections. It is unclear whether the initial decrease in circulating granulocytes observed in some patients may increase the risk of infections. IFN-induced leukopenia has not been associated with a higher incidence of infections in other cancer patients.[5]

Other B cell malignancies, including both low-grade lymphomas and multiple myeloma, have been shown to be sensitive to the antitumor effects of IFNα.[3-5] In contrast, advanced cases of intermediate or high-grade lymphomas or chronic lymphocytic leukemia have been resistant to IFNs.[4,18-21] It is presently unknown whether such differential sensitivity may be related to the stage of differentiation or the expression of cellular receptors in the malignant cells. In this regard, the expression in hairy cells of plasma cell-associated antigens is consistent with a stage of differentiation immediately preceding that of plasma cells.[22] Similarly, the expression of the Tac antigen, initially described as a marker of T lymphocytes, led Korsmeyer and collaborators[9] to suggest a unique stage of differentiation of hairy cells or a particular stage of activation. The sensitivity of cutaneous T cell lymphomas to IFNα could have in common with HCL the expression of this cell surface antigen.[23]

We conclude that rIFNαA is an effective method of treatment for all stages of HCL. rIFNαA can be reasonably indicated as initial treatment of newly diagnosed or untreated patients whose disease is progressing, although its use prior to splenectomy should still be reserved for patients participating in controlled clinical studies or for patients with a high surgical risk.

We are presently addressing further questions regarding duration of remissions, the need for prolonged maintenance therapy, the incidence of infections, and the impact on the survival of IFNα of >80 patients with HCL being followed at our institution.

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Thrombin Binding and Response in Platelets From Patients With Dyslipoproteinemias: Increased Stimulus–Response Coupling in Type II Hyperlipoproteinemia

By Joan T. Harmon, Narendra N. Tandon, Jeffrey M. Hoeg, and G.A. Jamieson

Platelets were obtained from patients with various hyperlipidemias [type II, type V, lecithin–cholesterol acyltransferase (LCAT) deficiency] and hypolipidemias (abetalipoproteinemia, Tangier disease) to ascertain relationships among plasma lipids, platelet lipids, thrombin binding and thrombin-induced platelet aggregation, and to compare these data with those previously obtained on stimulus–response coupling in platelets following in vitro modification of membrane microviscosity. Washed platelets were studied for their ability to bind ^{125}I-thrombin in the range of 10^{-10} to 10^{-6} mol/L (10 mU/mL to 100 U/mL) and to aggregate with thrombin at concentrations $<10^{-9}$ mol/L (100 mU/mL). The values for binding and aggregation in eight patients from six kindred with familial hypercholesterolemia, taken as a group, fell in the low normal range. If divided into two groups, patients with overt cardiovascular disease bound normal amounts of thrombin but were more responsive to it, whereas patients without overt cardiovascular disease bound lower amounts of thrombin but gave an aggregation response in the normal range. These results suggest that platelet hyperresponsiveness in familial hypercholesterolemia arises from an alteration in the coupling mechanism between thrombin binding and response such that platelets from patients with familial hypercholesterolemia are able to respond with lower receptor occupancy than is the case with normal platelets. Thrombin binding and aggregation were within normal ranges for platelets from abetalipoproteinemia patients ($N = 4$) and type V hyperlipoproteinemia ($N = 2$), although in the latter case the response appeared to be less at very low thrombin concentrations (<30 mU/mL). Thrombin binding was elevated in Tangier disease ($N = 3$) but with lower responsiveness at lower thrombin concentrations. Thrombin binding was also elevated in LCAT deficiency ($N = 2$), and one patient showed increased and another showed decreased aggregation responses. In general, increased plasma cholesterol levels resulted in increased stimulus–response coupling (type II), whereas increased triglyceride levels resulted in decreased coupling (type V, Tangier), and there was no apparent alteration in the coupling mechanism with overall reduction in plasma lipid levels as in abetalipoproteinemia.

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IT IS GENERALLY accepted that platelets play an important role in atherosclerosis and atherogenesis.[1,2] The high incidence of atherosclerosis and its thrombotic complications in certain types of hyperlipoproteinemias[3] has led to several studies of platelet activation in the dyslipoproteinemias.[4-9] Relatively few of these studies have used thrombin as the aggregating agent, and none has addressed the question of thrombin binding and stimulus–response coupling in platelets from patients with dyslipoproteinemias.

In normal platelets, activation by thrombin appears to involve binding of the agonist to surface receptors and a subsequent effector step leading to platelet activation and aggregation.[10,11] Three different types of binding sites have been identified[12]: a small number of sites (~50) of high affinity for thrombin [kilodalton (kd) ~ 0.3 nmol/L], a larger number of sites (~2,000) of moderate affinity (kd ~ 10 nmol/L), and a large number of sites (~600,000) of very low affinity (kd ~ 3 μmol/L). When platelet membrane microviscosity is increased by incubation in vitro with cholesterol-rich liposomes there is a corresponding increase in platelet aggregability and an increase in the number of thrombin receptors. The increase in number is accompanied by a decrease in affinity but no change in stimulus–response coupling[13]; conversely, a decrease in membrane microviscosity is accompanied by decreased aggregability and a decreased number of binding sites with increased affinity.

Elevations in the cholesterol–phospholipid ratio and, by extension, in the membrane microviscosity have been reported in type II hyperlipoproteinemias.[6] Because of the implications of these changes with respect to thrombin-induced activation and stimulus–response coupling, the present study was undertaken to ascertain the relationships among plasma lipids, platelet lipids, thrombin binding to platelets, and thrombin-induced platelet aggregation in humans as an extension of our previous studies on in vitro modification of membrane microviscosity. We have examined the in vivo effects of hyperlipidemia on platelet–thrombin interactions using platelets isolated from patients with increased concentrations of VLDL (type V hyperlipoproteinemia, $N = 2$), LDL (homozygous familial hypercholesterolemia, type II hyperlipoproteinemia, $N = 8$), and free cholesterol (lecithin–cholesterol acyltransferase [LCAT] deficiency, $N = 2$). In addition, platelets were studied from patients with the congenital hypolipidemic conditions: absence of VLDL and LDL (abetalipoproteinemia, $N = 4$) and the absence of HDL (Tangier disease, $N = 3$). Because these inborn errors of metabolism are rare, with <100 patients being known in any category, the above numbers constitute a reasonably representative sampling.

MATERIALS AND METHODS

Normal Volunteers

Control blood samples were obtained from normal volunteers who were either participating in metabolic turnover studies at the National Institutes of Health or were laboratory personnel. Their

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Table 1. Plasma Lipids of Patient Classes

Subjects	N	Total Cholesterol	VLDL	LDL	HDL	Triglycerides
Normal	15	167 ± 29	28 ± 17	90 ± 15	55 ± 14	123 ± 52
Type II	8	574 ± 164	41 ± 21	501 ± 150	34 ± 12	170 ± 102
Type V	2	292 ± 47	217 ± 41	50 ± 6	26 ± 1	2,237 ± 830
Tangier disease	3	93 ± 13	42 ± 14	49 ± 2	2.3 ± 0.6	422 ± 161
Abetalipoproteinemia	4	32 ± 6	1.5 ± 1.3	0	31 ± 5	10 ± 7
LCAT deficiency	2	142 ± 16	44 ± 27	92 ± 12	6 ± 2	522 ± 298

Values are given as the means ± the range ($N = 2$) or SD ($N > 2$). LCAT, lecithin-cholesterol acyltransferase.

mean age was 23.1 years (range 19 to 33 years). Of the 15 normal volunteers, 8 were men and 7 were women. At least one control sample was examined on each day that a patient sample was examined.

Patients

Patients with types II and V hyperlipoproteinemia demonstrated the typical clinical and biochemical criteria for these respective diagnoses.[14] The diagnoses of the following specific genetic diseases were based on standard criteria: homozygous familial hypercholesterolemia (HFH),[15] Tangier disease,[16] abetalipoproteinemia,[17] and LCAT deficiency.[18] The fasting plasma lipid and lipoprotein concentrations of the study patients are given in Table 1.

The LCAT-deficient patients were not on any medications (including aspirin). The patients with Tangier disease, abetalipoproteinemia, and type V hyperlipoproteinemia had not taken any lipid-lowering medications or aspirin for at least 6 weeks prior to blood sampling. The patients with HFH refrained from taking aspirin for at least 2 weeks prior to blood sampling and were taking the medications listed in Table 2 at the time of these studies.

Most of the patients with HFH were examined on at least two occasions. Patient Jo.P. was examined three times; patients V.G., Ja.P, D.P., and T.H. were each examined twice, although aggregation studies were not carried out on the two latter patients; and patients V.H., T.P., and L.R. were each examined once for both binding and aggregation.

Lipoprotein Quantitation

Blood was obtained from all study subjects in the supine position after a 12- to 14-hour overnight fast. After blood was collected in 0.01% EDTA, plasma was separated by centrifugation at 800 g for 30 minutes at 4 °C. Plasma total cholesterol and triglyceride concentrations were determined by an enzymic, colorimetric assay in a Gilford spectrophotometer, Model 3500 (Table 1). The HDL concentration was determined by subtracting the cholesterol concentration remaining after dextran sulfate precipitation from the total plasma cholesterol concentration.[19] Plasma ($d = 1.006$ g/mL) was ultracentrifuged for 18 hours at 39,000 rpm (4 °C) in 40.3 rotors (Beckman, Palo Alto, Calif), and the VLDL was separated from the other plasma lipoproteins by tube slicing. The cholesterol concentrations in the 1.006 g/mL infranatant were determined, and the VLDL and LDL cholesterol concentrations were calculated as standardized for the Lipid Research Clinics.[21] Platelet cholesterol and phospholipid contents were measured (Table 3) as described earlier.[13]

Platelet Preparation

Fresh blood (50 mL) anticoagulated with citrate/phosphate/dextrose was obtained from individual patients and normal volunteers. Platelet-rich plasma (PRP) was obtained by centrifugation at 800 g for ten minutes at 22 °C. The pH of the PRP was adjusted to 6.5 with citric acid and the platelets were sedimented by centrifugation at 1,600 g for ten minutes. The platelets were resuspended and washed in citrate wash buffer (11 mmol/L of dextrose; 128 mmol/L of NaCl; 4.26 mmol/L of Na_2HPO_4; 4.77 mmol/L of trisodium citrate; and 2.35 mmol/L of citric acid, pH 6.5) supplemented with 0.35% bovine serum albumin as previously described.[13] After three washes, the platelets were either resuspended in Tris-binding buffer (136 mmol/L of NaCl; 25 mmol/L of Tris; and 0.6% polyethylene glycol 6,000, pH 7.4) for thrombin-binding studies or in modified Tyrode's buffer (136 mmol/L of NaCl; 5.5 mmol/L glucose; 2.7 mmol/L of KCl; 0.07 mmol/L of Na_2HPO_4; and 0.01 mmol/L of $NaHCO_3$, pH 7.4) for studies on thrombin-induced aggregation.

Thrombin-induced Aggregation

Aggregation of washed platelets in response to increasing concentrations of thrombin (10 to 100 mU/mL) was measured in a Payton aggregometer (Payton Associates, Buffalo) at 37 °C as previously described.[13] Dose-response curves were constructed for each individual, and the thrombin concentration required for half-maximal aggregation was determined. Data were normally distributed and, where more than two patients were available, significance was calculated by Student's t test.

Thrombin Binding Assay

Platelets (4×10^8/mL) were incubated with ^{125}I-thrombin (10^{-10} mol/L), without or with increasing concentrations of unlabeled thrombin (10^{-10} to 10^{-6} mol/L), in Tris binding buffer for 10 minutes at 22 °C as previously described.[13] Under these conditions, the thrombin binding reached steady state, and thrombin degrada-

Table 2. Clinical Features of Homozygous Familial Hypercholesterolemic Patients

Patient	Age	Sex	LDL Receptor Activity (%)	Treatment	Cardiovascular Disease
D.P.	7	M	4	Portacaval shunt	Femoral bruits and cardiac catheterization
V.G.	6	F	1	Portacaval shunt	None evident by exercise stress test
Ja.P.	22	F	29	Diet only	Carotid femoral bruits and cardiac catheterization
Jo.P.	20	F	24	Cholestyramine	None evident by cardiac catheterization
T.P.	26	M	26	Cholestyramine	Femoral bruits and cardiac catheterization
V.H.	22	F	6	Diet only	Femoral bruits and cardiac catheterization
L.R.	26	F	14	Diet only	None evident by stress test
T.H.	13	F	11	Portacaval shunt	None evident by cardiac catheterization

Table 3. Platelet Lipid Composition

Subjects	C*	PL*	C:PL†
Normal (12)	1,129 ± 217	3,505 ± 670	0.64 ± 0.066
Type II (11)	1,093 ± 235	3,096 ± 614	0.71 ± 0.084‡
Type V (2)	1,140 ± 60	4,000 ± 300	0.58 ± 0.021
Tangier disease (1)	980	3,250	0.57
Abetalipoproteinemia (4)	1,287 ± 277	3,960 ± 315	0.65 ± 0.11
LCAT deficiency (2)	1,150 ± 30	3,875 ± 125	0.60 ± 0.034

C, cholesterol; P, phospholipid; C:PL, cholesterol-phospholipid ratio.

Numbers in parentheses are the numbers of different determinations; sufficient platelets were available for lipid assay with only one of the three Tangier patients. Four of the type II patients (T.H., Ja.P., Jo.P., and V.G.) were assayed on two occasions.

*Values are given in micrograms per 10^{10} platelets ± range (N = 2) or SD (N > 2).

†Values are given as the molar ratios ± range (N = 2) or SD (N > 2).

‡Significantly different from normal ($P < .05$).

tion was <5% as determined by the amount of radioiodine soluble in 5% trichloroacetic acid.

Data Analysis

Data from binding experiments were analyzed by a weighted nonlinear least-squares curve fitting program (LIGAND)[22] as previously described.[13] Data were analyzed by assuming a model of three independent binding sites without a nonsaturable component since this model gives the best fit as determined by the least sum of squares method (*F* test comparison).[13] Comparisons were made between data sets and statistical significance was determined using a computer program, RS1 (BBN Research Systems, Cambridge, Mass). This program compares data to test for normality (Kolmogorov-Smirnov or Wilk-Shapiro methods) and, depending on this analysis, uses a parametric procedure (*t* test, either one sample or paired or *F* test for equality of variance) or nonparametric procedure (Wilcoxon signed rank test or Ansari-Bradley test for equality of dispersions) to determine significance.

RESULTS

Lipid and Lipoprotein Levels

The values for plasma lipids in the patients correspond with values previously obtained in defining these disease classes (Table 1): specifically, the type II patients had elevations in total and LDL cholesterol concentrations; type V had elevations in concentrations of VLDL cholesterol and triglycerides; the three patients with Tangier disease had low concentrations of total plasma and HDL cholesterol but elevated triglyceride concentrations; and the four patients with abetalipoproteinemia had low concentrations of total cholesterol (virtually all of which was in HDL) and low triglyceride concentrations. The LCAT-deficient patients had elevated concentrations of triglycerides but low concentrations of HDL cholesterol. Because platelet reactivity has been correlated with membrane microviscosity,[13,23] which is

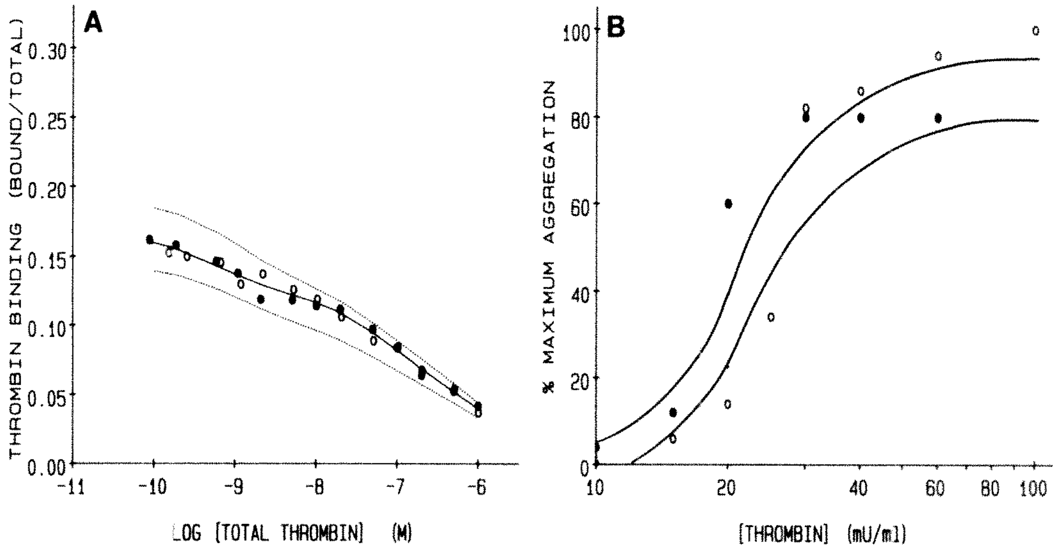

Fig 1. Thrombin binding and thrombin-induced aggregation of platelets from a normal volunteer on two different occasions. Left: thrombin binding isotherms. Dotted lines denote mean ± 2 SD of results obtained using platelets from 15 normal volunteers. Data points are the mean of triplicate determinations in the first experiment (solid circle) and in the second experiment 6 weeks later (open circle). Solid line represents the best-fitting line generated by computer analysis assuming a model of three independent binding sites for the data obtained in the two separate experiments. Right: thrombin-induced aggregation response curves; solid lines denote mean ± 1 SD of results obtained using platelets from 15 normal volunteers. Data points represent the percentage of maximum aggregation obtained in the first experiment (solid circle) and in the second experiment (open circle). In each experiment, binding and aggregation studies were performed on the same day.

proportional to the cholesterol–phospholipid ratio (C:PL),[23] analytical data for platelet cholesterol, phospholipid and the C:PL ratio are given in Table 3. There were no significant differences from normal controls in the platelet cholesterol levels and platelet phospholipid levels in any of the patient groups. However, there was a significant difference ($P < .05$) between the platelet C–PL ratio of normal volunteers and the platelet C–PL ratio of type II hyperlipoproteinemia patients but not with those of the other patient groups.

Thrombin Binding and Aggregation

Normal Volunteers

On each day that a patient sample was studied, at least one sample from a normal volunteer was also studied. On several occasions, the same normal volunteer was used to determine between-assay variations. Results indicated no significant difference in the thrombin-binding isotherms obtained on two separate occasions and that the thrombin-induced dose–response curves (performed on the same days as the binding assay) were reproducible within the limits of this type of assay (Fig 1). No differences were observed dependent on the sex of the normal volunteer with regard to thrombin binding or thrombin-induced aggregation (data not shown).

Patients

Type II. Comparison of the thrombin-binding isotherms of platelets from eight type II (HFH) patients with those of normal controls showed that the data were within the normal range (±2 SD) albeit at the lower end. Similarly, the concentration dependence of aggregation showed an apparently random spread in relation to the aggregation data obtained with normal controls. However, closer examination showed differences in these parameters if the data were analyzed on the basis of the presence or absence of cardiovascular disease. The clinical features of these patients are given in Table 2. Four of the patients did not have overt cardiovascular disease, and their platelets bound lower amounts of thrombin than did those of normal controls (Fig 2A); however, the observed aggregation was within the normal range (Fig 2B), and the thrombin concentration required for half-maximal aggregation (24 ± 5 mU/mL in eight determinations on the four patients) was identical with that of normal controls (23 ± 7 mU/mL; $N = 14$). On the other hand, four of the patients had cardiovascular disease, as indicated by femoral bruits and confirmed by cardiac catheterization. The thrombin-binding isotherm for these patients fell within the range of normal controls (Fig 2C), but platelets from these patients were hyperaggregable, as shown by the leftward shift of the concentration-dependence data (Fig 2D) and the fact that the thrombin concentration for half-maximal aggregation was decreased to 16 ± 3 ($P = 0.089$; $N = 4$). That is, patients without cardiovascular disease bound lower amounts of thrombin than did normal control subjects but gave an aggregation response in the normal range whereas patients with cardiovascular disease bound normal amounts of thrombin but showed an increased aggregation response.

Computer analysis of the thrombin-binding isotherms resulted in the calculated binding parameters shown in Table 4. The amount of thrombin bound at a particular site is a function of the number of sites (R) and the affinity (K) of that site for thrombin. Thus, the product of these parameters (KR) is a better reflection of the amount of ligand bound than is either parameter (K or R) alone. It is the theoretical maximum binding to a particular site at infinite dilution of the labeled ligand and can be more precisely determined than can either the affinity or the receptor number.[29] There is a statistically significant decrease in the KR parameters for each of the three binding sites on platelets from HFH patients without cardiovascular disease in comparison to these parameters for platelets from normal volunteers (Table 5). Thus, there appears to be a decrease in binding to all three binding sites on platelets from HFH patients without cardiovascular disease, whereas normal levels are observed following the onset of cardiovascular disease.

Type V. Platelets from the two patients with type V hyperlipoproteinemia showed a thrombin-binding isotherm similar to that of normal platelets (Fig 2E). However, there was a significant increase in the K_1R_1 parameter, suggesting an increase in binding to the high-affinity binding site. These platelets appeared to be less responsive to low thrombin concentrations (<30 mU/mL) than those of normal volunteers but responded normally to higher thrombin concentrations (Fig 2F), and the thrombin concentration for half-maximal aggregation was calculated to be 30 ± 1 mU/mL.

LCAT deficiency. Only two patients with LCAT deficiency were available for study. The thrombin-binding isotherm for these two patients (Fig 2K) fell at the +2 SD line for normal controls in the range of the low-affinity receptor (thrombin concentration $> 10^{-7}$ mol/L) but was ~50% above the range of normal controls at lower thrombin concentrations, indicating greater binding of thrombin to the high-affinity and moderate-affinity sites. There was a significant increase in KR parameters for each of the three binding sites. The aggregation patterns for these two patients (Fig 2L) were difficult to reconcile with the thrombin binding data: one patient showed an approximately threefold greater sensitivity to thrombin than did normal volunteers, as indicated by aggregation response points, which all fell to the left of the normal response curve. Conversely, the other patient showed a reduced response to thrombin, with all of the aggregation points falling to the right of the normal response curve. All data taken together indicated that the thrombin concentration for half-maximal aggregation was 24 ± 12 mU/mL.

In addition to the above studies, thrombin binding and aggregation have also been examined in washed platelets from patients suffering from the hypolipoproteinemias, Tangier disease, and abetalipoproteinemia.

Tangier disease. Platelets from the three patients with Tangier disease showed a thrombin-binding isotherm that was >2 SD above the mean of 15 normal controls over the thrombin concentrations corresponding to high-affinity and moderate-affinity receptors, but the difference appeared to be less marked at high thrombin concentrations corresponding to the low-affinity receptor (Fig 2G). The increase in binding is reflected by significant increases in the K_1R_1 and K_2R_2 parameters. The aggregation data suggested that Tan-

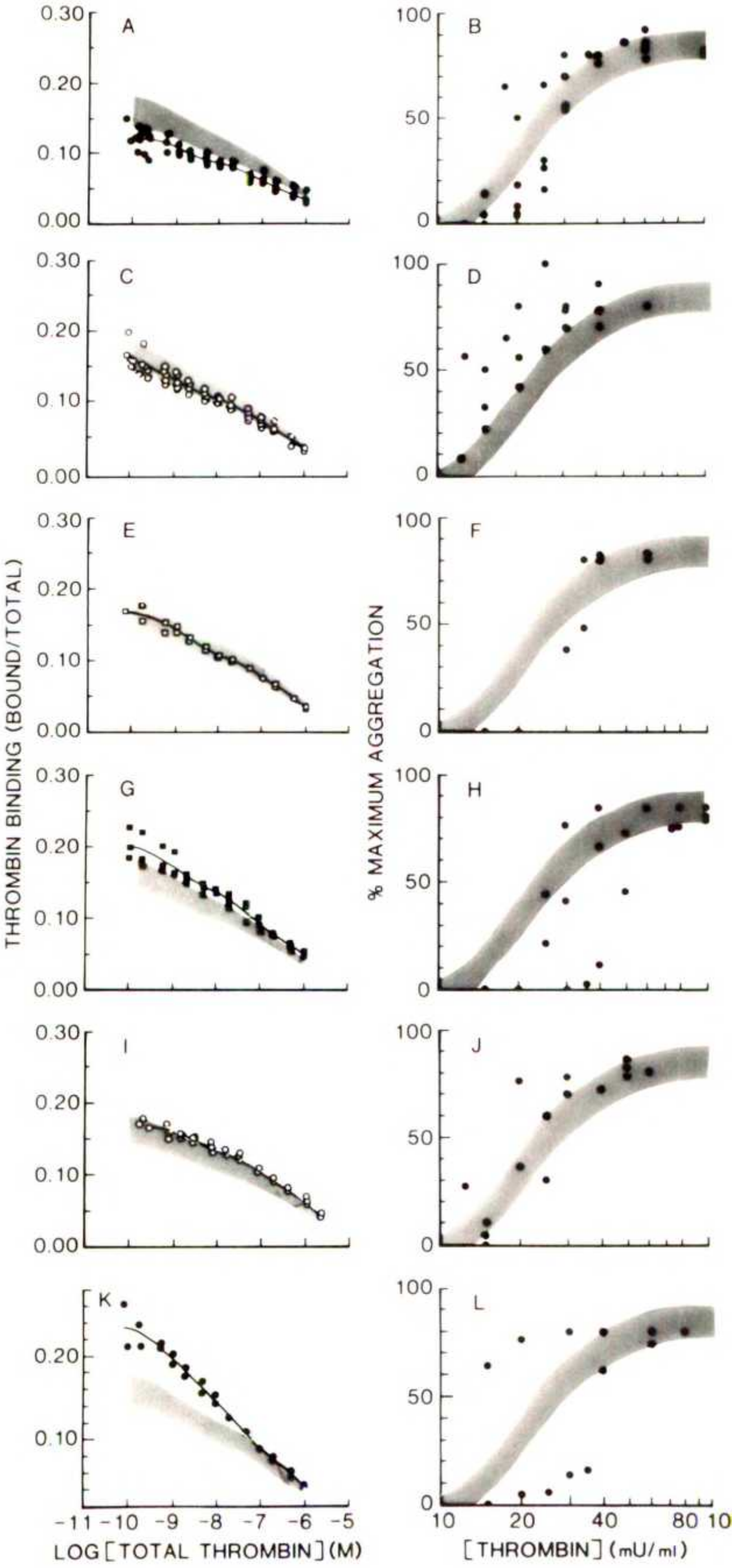

Fig 2. Thrombin binding and thrombin-induced aggregation of platelets obtained from patients with dyslipoproteinemias. Left column of figures (A,C,E,G,I, and K): thrombin-binding isotherms; shaded area denotes mean ± 2 SD of results obtained using platelets from 15 normal volunteers. Right column of figures (B,D,F,H,J, and L): thrombin-induced aggregation response data; shaded area denotes mean ± 1 SD of results obtained using platelets from 15 normal volunteers. Data points were obtained with platelets from HFH patients without cardiovascular disease (A and B), homozygous familial hypercholesterolemia (HFH) patients with cardiovascular disease (C and D), patients with type V hyperlipoproteinemia (E and F), patients with Tangier disease (G and H), patients with abetalipoproteinemia (I and J), and patients with lecithin-cholesterol acyltransferase (LCAT) deficiency (K and L).

Table 4. Thrombin-Binding Parameters

Subjects	K_1 (nmol/L)	K_2 (nmol/L)	K_3 (umol/L)	R_1*	R_2*	R_3*
Normal	0.84 ± 0.3	51 ± 20	1.2 ± 0.3	89 ± 30	4,900 ± 2,000	120,000 ± 12,000
Type II						
No disease	0.73 ± 0.6	40 ± 30	1.6 ± 0.7	53 ± 50	3,100 ± 2,000	120,000 ± 30,000
Disease	0.45 ± 0.2	51 ± 30	1.4 ± 0.6	57 ± 30	5,000 ± 3,000	120,000 ± 30,000
Type V	1.1 ± 0.5	81 ± 70	0.90 ± 0.5	150 ± 60	6,800 ± 8,000	84,000 ± 10,000
Tangier	0.73 ± 0.5	67 ± 40	3.0 ± 3	110 ± 80	10,000 ± 8,000	280,000 ± 220,000
Abetalipoproteinemia	0.39 ± 0.4	33 ± 10	0.91 ± 0.2	31 ± 30	3,900 ± 2,000	120,000 ± 10,000
LCAT deficiency	0.72 ± 0.6	23 ± 16	1.1 ± 0.3	140 ± 100	3,900 ± 2,000	130,000 ± 20,000

LCAT, lecithin-cholesterol acyltransferase.

Affinities for each binding site are given as the dissociation constant.

*Total number of receptors is given as sites per platelet.

gier disease platelets were somewhat less responsive to thrombin than were those of normal control subjects, and that this difference was most marked at lower thrombin concentrations (Fig 2H). This difference was also indicated in the thrombin concentration required for half-maximal aggregation (34 ± 10 in three determinations; $P = .041$).

Abetalipoproteinemia. The thrombin-binding isotherm for the four patients with abetalipoproteinemia fell almost exactly on the ±2 SD line of normals (Fig 2I). There were no significant differences from normal volunteers in the KR parameters. Aggregation data were scattered, with no detectable differences from data for normal subjects (Fig 2J), and a half-maximal aggregation value of 20 ± 6 mU/mL ($N = 4$) was calculated.

Correlations. The positive correlation between total plasma cholesterol concentration and the cholesterol content of low-density lipoprotein (LDL) has been reported in many studies. We have also observed this correlation in the present study. Other correlations which were found to be statistically significant ($P < .01$) in the present work include: (a) the positive association between the platelet C:PL ratio and the total plasma concentration of cholesterol or the cholesterol concentration in the LDL, and (b) the indirect correlation between the platelet C:PL ratio and the total amount of thrombin bound to the platelet ($K_1R_1 + K_2R_2 + K_3R_3$). There is also an indirect correlation ($P = .064$) between the percentage of LDL receptor activity on fibroblasts and the concentration of thrombin necessary to elicit a 50% aggregation response with platelets from HFH patients. The physiologic relevance of these correlations has not been determined.

DISCUSSION

Several studies have reported on the aggregability of platelets in dyslipoproteinemias. However, agonist binding to platelets and stimulus–response coupling have not been studied, and in only a few cases has platelet responsiveness to thrombin been examined. A further variable in aggregation studies has been the use of platelets in plasma or gel-filtered platelets, which has prevented differentiation of intrinsic aspects of platelet response from secondary effects due to plasma components, especially lipoproteins that are known to interact with platelets and affect platelet function.[8,24-26] To clarify several of these points, we studied the binding of thrombin and thrombin-induced aggregation using washed platelets from a variety of patients with hyperlipoproteinemias and hypolipoproteinemias.

The cholesterol and phospholipid analysis of platelets from patients with LCAT deficiency and type V hyperlipoproteinemia do not appear to have been previously reported. For normal control subjects and HFH patients, our values are in good agreement with those previously reported,[5] as is the case for the single patient with Tangier disease whose platelet lipids were analyzed.[6] The values previously reported for cholesterol content in patients with abetalipoproteinemia (2,390 ± 106 $\mu g/10^{10}$ platelets; $N = 2$)[6] are almost twice those found in the present work (1,287 ± 277; $N = 4$) and yielded an unusually high C:PL ratio of 1.47: this value may be questioned since it is much higher than C:PL ratios found in any of the other dyslipoproteinemias which range from ~0.6 in type V to ~0.7 in HFH patients and is even higher than values reported for platelets artifically enriched in

Table 5.

Subjects	K_1R_1	K_2R_2	K_3R_3
Normal	0.071 ± 0.0005	0.063 ± 0.0006	0.066 ± 0.001
Type II			
No disease	0.048* ± 0.002	0.051* ± 0.001	0.051* ± 0.002
Disease	0.084 ± 0.01	0.065 ± 0.01	0.057 ± 0.01
Type V	0.088* ± 0.001	0.056 ± 0.008	0.062 ± 0.01
Tangier	0.10* ± 0.003	0.10* ± 0.004	0.062 ± 0.007
Abetalipoproteinemia	0.053 ± 0.02	0.080 ± 0.008	0.089 ± 0.01
LCAT deficiency	0.13* ± 0.006	0.11* ± 0.005	0.082* ± 0.002

LCAT, lecithin-cholesterol acyltransferase.

Values are given as the mean ± SE and are the product of the association constant $[(mol/L)^{-1}]/(mol/L)$ and the number of binding sites (M).

*Significantly different from normals ($P < .0005$).

cholesterol by incubation with cholesterol-rich liposomes (~0.9).[13,23]

Platelets from HFH patients have previously been reported to show increased rate and amplitude of aggregation with moderate concentrations of thrombin (500 mU/mL), whether as platelet-rich plasma or as plasma-free gel-filtered platelets,[8] which has been taken to indicate an intrinsic platelet abnormality in hyperlipoproteinemia.

In the present work, we were not able to show any abnormalities in thrombin binding or aggregation of washed platelets from HFH patients as a whole. However, useful analysis was achieved by dividing the patients into two groups, those with and those without overt cardiovascular disease. Platelets from HFH patients with cardiovascular disease bound normal amounts of thrombin but were more responsive to the bound thrombin than were normal platelets. On the other hand, platelets from HFH patients without cardiovascular disease bound lower amounts of thrombin than did normal platelets but still gave aggregation responses in the normal range. These results suggest that in both platelet groups there is an alteration in the coupling mechanism which links the thrombin-binding sites to the response system which enables thrombin, with these platelets, to elicit a response with lower receptor occupancy than is possible in normal platelets. The separation of the patients into two groups shows that significant clinical changes correlate with measurable biochemical changes which are obscured if the data are taken as a whole.

All HFH patients eventually develop cardiovascular disease. Thus, the phenotypic characteristic of the platelet-thrombin interaction in these patients is probably that seen in the group prior to the onset of cardiovascular disease, namely, a decreased binding of thrombin but an increased responsiveness to the thrombin bound so that normal aggregation results. The onset of cardiovascular disease could result from a shift in this phenotypic characteristic to the second situation, in which normal amounts of thrombin are being bound and the increased responsiveness of HFH patients is manifesting itself as increased aggregability. However, there appear to be other, possibly more plausible, explanations: damage to the internal surface of blood vessels could result in a mild degree of platelet activation, and this activation could itself result in a synergistic response during further exposure to thrombin. Alternatively, platelets from hypercholesterolemic patients with cardiovascular disease have been reported to have a shorter survival time than that of platelets from hypercholesterolemic patients without cardiovascular disease.[27] Thus, the shift in the binding isotherm and in the dose–response curve seen with platelets from HFH patients with cardiovascular disease could be the result of an increased population of younger platelets, which have been reported to respond to lower concentrations of agonists,[28] rather than the result of inherent differences in platelets in the two patient groups. A corollary of this latter interpretation is that the number of thrombin receptors on platelets would be expected to be inversely proportional to platelet age.

These results using platelets from patients with HFH differ from those obtained in studies on thrombin binding and responsiveness following in vitro manipulation of the platelet C:PL ratio. In the latter case, the hyperresponsiveness arises from increased expression of thrombin receptors without any change in stimulus–response coupling[13] whereas, in the present situation, that hyperresponsiveness in both groups of HFH patients is due to an increase in stimulus–response coupling.

Patients with type V hyperlipoproteinemia, Tangier disease, or LCAT deficiency have varying lipoprotein concentrations, but all have an increased plasma triglyceride concentration. The platelets from patients in these three disease groups all showed increased binding of thrombin to the high-affinity binding site (K_1R_1, Table 5) and demonstrated a reduced sensitivity to low concentrations of thrombin. These data suggest that elevated plasma triglyceride concentrations may cause the uncoupling of the high-affinity thrombin binding site from the response system. However, reduced plasma cholesterol levels and triglyceride levels in platelets from abetalipoproteinemia patients do not appear to alter either interaction with thrombin or thrombin responsiveness, suggesting that stimulus–response coupling is similar to that in platelets from normal volunteers. The recent studies of Aviram et al[8] offer further evidence that triglyceride levels (associated with chylomicrons) are inhibitory to platelet function. Their studies report a normal response of gel-filtered platelets from type V hyperlipoproteinemic patients to 200 mU/mL of thrombin, and the results are very similar to those reported here. However, they did not examine the responsiveness of these platelets to lower thrombin concentrations (<30 mU/mL), which in our study appeared to demonstrate a reduced platelet responsiveness. Further studies are necessary to define clearly the effects of plasma triglyceride levels on platelet-thrombin interactions.

In conclusion, we used platelets from patients with various, rare, inborn errors of lipid metabolism to extend our earlier studies which showed that in vitro manipulation of C:PL ratios resulted in increased aggregation due to increased expression of thrombin receptors with no alteration in stimulus–response coupling.[13] Three major conclusions may be drawn from the present work: (*a*) In vivo elevations of C:PL ratios in both groups of HFH patients resulted in increased aggregation response due to increases in stimulus–response coupling; (*b*) this increased stimulus–response coupling occurred in patients both with and without overt cardiovascular disease; and (*c*) normal C:PL ratios in other dyslipoproteinemic patients are not necessarily accompanied by normal thrombin binding or normal aggregation response. Finally, these studies show that in vitro manipulation of C:PL ratios, while perhaps providing useful information on membrane function, does not provide an adequate model for physiopathologic situations.

ADKNOWLEDGMENT

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Accurate and Independent Measurement of Volume and Hemoglobin Concentration of Individual Red Cells by Laser Light Scattering

By Narla Mohandas, Y.R. Kim, D.H. Tycko, J. Orlik, J. Wyatt, and Warren Groner

Cell volume (MCV) and hemoglobin concentration (MCHC) are the red cell indices used to characterize the blood of patients with anemia. Since the introduction of flow cytometric methods for the measurement of these indices, it has generally been assumed that the values derived by these instruments are accurate. However, it has recently been shown that a number of cellular factors, including alterations in cellular deformability, can lead to inaccurate measurement of cell volume by these automated instruments. Because cell hemoglobin concentration and hematocrit are computed from the measured values of cell volume, accuracy of these indices is also compromised by inaccurate determination of cell volume. A recently developed experimental flow cytometric method based on laser light scattering, which can independently measure volume and hemoglobin concentration, has been used in the present study to measure MCV and MCHC of density-fractionated normal and sickle red cells, hydrated and dehydrated normal red cells, and various pathologic cells. We found that the new method accurately measures both volume and hemoglobin concentrations over a wide range of MCV (30 to 120 fL) and MCHC (27 to 45 g/dL) values. This is in contrast to currently available methods in which hemoglobin concentration values are accurately measured over a more limited range (27 to 35 g/dL). In addition, as the experimental method independently measures volume and hemoglobin concentration of individual red cells, it allowed us to generate histograms of volume and hemoglobin concentration distribution and derive coefficient of variation for volume distribution and standard deviation of hemoglobin concentration distribution. We have been able to document that volume and hemoglobin concentration distributions can vary independently of each other in pathologic red cell samples.

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CELL VOLUME AND HEMOGLOBIN CONCENTRATION are the red cell indices used to characterize the blood of patients with anemia. Since their introduction by Wintrobe in the early 1930s, they have formed the basis for classification of most anemias.[1,2] When originally introduced, both mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) were derived from manually measured values of hemoglobin, red cell count, and packed cell volume. Currently used flow cytometric methods directly measure cell volume and derive a value for MCHC from this measured cell volume. The commonly used flow cytometric methods derive cell volume either by measuring changes in electrical resistance (Coulter principle) or by measuring the amount of light scattered by individual red cells flowing through a narrow sensing aperture. In instruments based on the Coulter principle, single red cells traverse a small aperture across which an electric field is applied. Because red cells are extremely poor conductors of electricity compared with the diluent, they cause a measurable change in electrical resistance during passage through the aperture. As increased resistance is nearly proportional to the volume of conducting medium displaced, the measurement of resistive pulses generated by flowing cells provides a measure of cell volume. In instruments based on light scattering, as single red cells pass through a narrow beam of light, they diffract light. Because the amount of light scattered is related to cell volume, measurement of forward light scattered at a defined angular interval provides a measure of cell volume. These flow cytometric methods provide accurate measurements of cell volume for normal red cells. However, it has recently been shown that a number of other cellular factors besides volume can influence the measurement[3-9] and that the volume measurements on certain pathologic red cells may be inaccurate. One of the cellular factors that affect volume measurement in flow systems is cell hemoglobin concentration, a quantity that is known to vary substantially among individual red cells in both normal (27 to 40 g/dL) and pathologic (25 to 50 g/dL) blood samples.[10-14] In the electrical sizing instruments, the magnitude of resistive pulse generated by a cell depends not only on its volume, but also on its form (shape factor), which is determined by the extent of deformation it undergoes while traversing the orifice.[5-7] Because cytoplasmic viscosity, determined by hemoglobin concentration, greatly influences cell deformation, cell volume measurements are affected by hemoglobin concentrations in this system. In the light-scattering system, as the hemoglobin concentration of a cell determines its refractive index, and hence the amount of light the cell scatters, the unknown hemoglobin concentration leads to an uncertainty in the volume to be associated with a given scatter signal. As the measured value of MCV by both systems is used to compute hematocrit and MCHC, the accuracy of these indices is also compromised by inaccurate determination of MCV.

We have developed a flow cytometer-based laser light-scattering method that directly measures both volume and hemoglobin concentration of individual red cells and thus permits independent measurement of these two red cell indices.[15] In this study, we have applied this experimental method to measure the MCV and MCHC of density-fractionated normal and sickle red cells, hydrated and dehydrated normal red cells, and various pathologic red cells. We find that the method accurately measures the MCV and MCHC of red cells over a large range of values for both these indices. In addition, the method, by providing us with the distributions of both cell volume and hemoglobin concentra-

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tion, has enabled us to distinguish between variations in both cell volume and cell hemoglobin concentration of red cells in a given blood sample.

MATERIALS AND METHODS

Blood samples were obtained from healthy volunteer donors, from patients with sickle cell anemia, and from a randomly selected group of hospitalized patients. In order to extend the range of red cell volumes measured during this study, blood was also obtained from two other mammalian species, rabbit and sheep.

Red cells with defined cell densities. Whole blood was separated on discontinuous Stractan density gradients to obtain red cell populations with narrowly defined ranges of cell density, and hence cell hemoglobin concentration.[10,11,13] An eight-step discontinuous Stractan density gradient with densities ranging from 1.070 to 1.105 g/mL in 0.0045-g/mL increments was used for separation of normal red cells. Thrice-washed red cells were layered on top of the gradient and spun in an L8-55 ultracentrifuge (Beckman, Palo Alto, Calif) for 45 minutes at 25,000 rpm. Red cell subpopulations accumulating at various density interfaces were harvested, washed twice with phosphate-buffered saline (PBS: 10 mmol/L phosphate, 290 mosm/kg, pH 7.4), and resuspended in PBS at approximately 40% hematocrit for volume and hemoglobin concentration determinations.

Hydrated and dehydrated normal red cells. The water content of normal red cells was varied by diluting or enriching the extracellular concentration of monovalent cations (Na^+ or K^+) in the presence of the permeabilizing antibiotic nystatin, as described previously.[16,17] Briefly, incubation with nystatin (30 μg/mL) at 0 °C permitted equilibrium of Na and K across the membrane at the prevailing osmotic concentration. Subsequent removal of nystatin by washing at room temperature with media having the same ion concentrations restored the normal permeability barrier. Then, when the cells were resuspended in isotonic (290 mosm/kg) PBS, they underwent shrinkage or swelling, depending on whether the osmolality during nystatin treatment was hypotonic or hypertonic, respectively. The extent of water loss or gain was consistent with deviation from normal osmolality during ion equilibration. Independent confirmation of the abnormal water content of the nystatin-treated cells was obtained from measurements of changes in osmotic fragility and cell density.[8]

Manual MCV and MCHC measurements. The hematocrit was measured using microhematocrit tubes that were centrifuged for ten minutes at 11,500 rpm in a microhematocrit centrifuge (International Equipment, Needham Heights, Mass).[18] The lengths of the packed red cell mass and total blood column were measured using a microscope stage micrometer at 50× magnification. Plasma trapping in the packed cell mass was quantitated using ^{125}I bovine serum albumin.[19,20] The corrected hematocrit was derived as measured hematocrit X (1 − trapped plasma fraction). Whole blood hemoglobin was determined according to the procedure described by van Kampen and Zijlstra.[21] The red cell counts were determined using a Coulter Electronics ZBI counter (Coulter Electronics, Hialeah, Fla). Manual MCV values were obtained by dividing corrected microhematocrit values by corresponding RBC counts, and manual MCHC values were computed by dividing measured hemoglobin values by corresponding corrected hematocrit values.

One might argue that variations in trapped plasma might render spun hematocrits inaccurate over a wide range of cellular hydration, and thus introduce errors in MCV and MCHC values derived by the manual method. In this context, it is important to point out that during this study we corrected for plasma trapping, and that the trapped plasma constituted no more than 3% for any of the samples, including oxygenated sickle cells. These low values for plasma trapping for sickle red cells are consistent with previous findings of Usami et al.[22]

Electronic aperture impedance volume measurements. Red cell indices were also determined on the Coulter S-plus (Coulter Electronics) instrument for various blood samples. The instrument was calibrated in the manner recommended by the manufacturer. Measurements were performed in duplicate.

Light-scattering system for measuring cell volume and hemoglobin concentration. The experimental measuring system used during this study consisted of a flow cytometer that simultaneously measured cell volume (V) and hemoglobin concentration (HC) of individual isovolumetrically sphered red blood cells. The theoretical basis for these measurements, as well as the optical and signal processing systems used to collect the data, have been described elsewhere.[15] Figure 1 shows a schematic diagram of the flow cytometric system. Briefly, the major components of this system are a helium–neon laser monochromatic light source, a sheathed-stream flow cell, an optical detection system that measures scattered-light intensity at two angles, S_1 and S_2. An electronic data-processing system, based on the Mie scattering theory, derives values for cell volume and refractive index for each individual red cell from the two measured scattered-light intensities. As red cell refractive index is a linear function of hemoglobin concentration, the derived refractive index is converted to cell hemoglobin concentration. The relationship among scatter signal intensities S_1, S_2, V, and HC are shown in Fig 2. With these tables, accurate and precise values of V and HC for each red cell in a blood sample are determined and histograms of V and HC are constructed. MCV, MCHC, width of volume distribu-

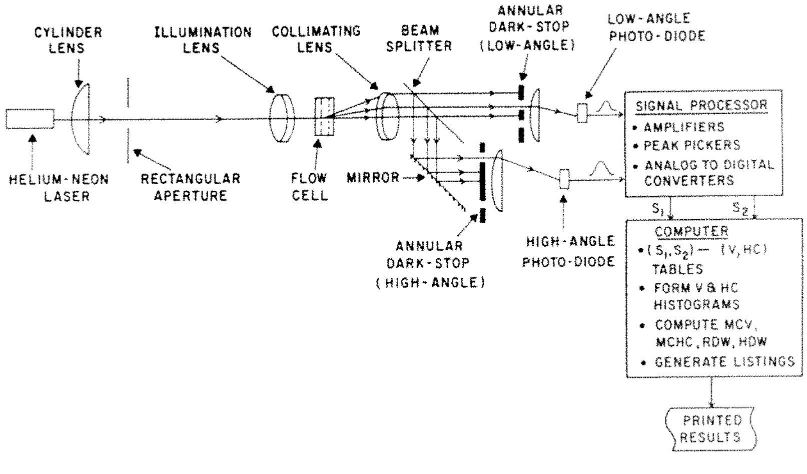

Fig 1. Schematic diagram of the measurement system. A 20 × 150-μm rectangular cross section of the flow cell is illuminated by the laser light source. Dilute red cell suspension driven by a standard peristaltic pump flows through the sheathed-stream flow cell at a linear velocity of approximately 1 m/sec. Sample stream diameter is approximately 35 μm. Measurements began 50 seconds after mixing of a 10-μL blood sample with 5 mL of sphering agent. Data were acquired for a period of ten seconds during which between 5,000 and 10,000 individual red cells were measured. A two-dimensional (S_1, S_2) pulse height distribution was formed for each sample from which volume and hemoglobin concentration histograms were generated.

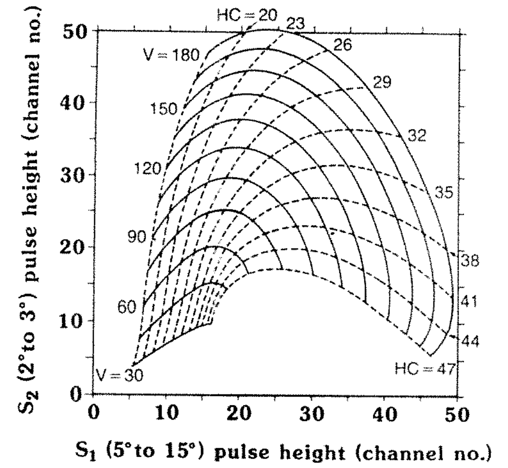

Fig 2. Mapping between (S_1, S_2) and volume (V) and hemoglobin concentration (HC). Solid curves are constant-V contours, labeled in femtoliters. Dashed curves are constant-HC contours labeled in g/dl.

tion (RDW), and width of hemoglobin concentration distribution (HDW) are computed from the histograms. RDW is derived as the coefficient of variation of the volume histogram, while HDW is derived as the standard deviation of the hemoglobin concentration histogram. A typical output listing from the measuring system on a normal blood sample is shown in Fig 3.

Red cells for volume and hemoglobin concentration measurement were prepared by mixing 10 μL of whole blood with 5 mL of sphering agent. The sphering agent used in these studies consisted of PBS (290 mosm/kg, pH 7.4) with 1 mg/dL sodium dodecylsulfate and 0.1% glutaraldehyde. Because Mie light-scattering theory can be directly applied only to particles with spherical geometry, it is necessary to sphere and fix the red cells before measurement. It has been shown that the rate of red cell sphering is appreciably faster than the time required to fix the cells, and that the red cells are indeed isovolumetrically sphered and fixed in this reagent.[23] The sphered and fixed red cells were aspirated through the sample stream of the flow cell at a velocity of approximately 1 m/sec. The measurements began 50 seconds after mixing of blood sample with the reagent, and light-scatter data were acquired for a period of ten seconds during which between 5,000 and 10,000 individual red cells were measured. Replicate measurement of a normal blood sample produced a mean of 90 fL with a standard deviation of 0.7 fL for MCV and a mean of 34.5 g/dL with a standard deviation of 0.4 g/dL for MCHC.

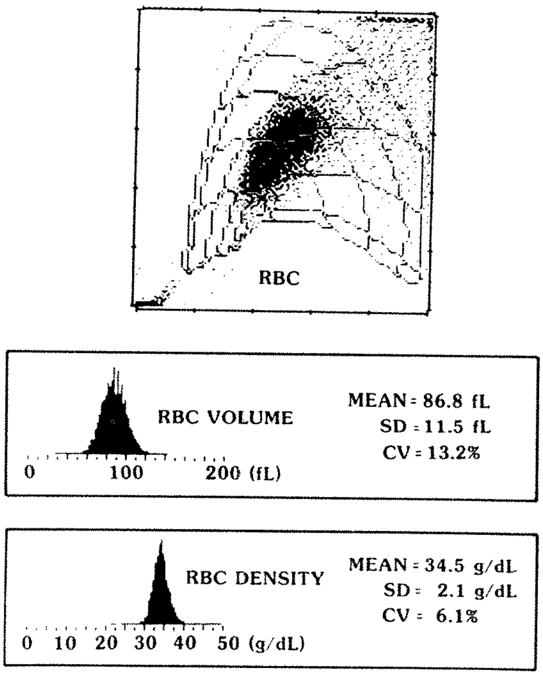

Fig 3. Sample output from data-processing subsystem. The scatter plot represents the two-dimensional (S_1, S_2) pulse height distribution. The (S_1, S_2) → (V, HC) mapping is overlayed on each scatter plot to aid interpretation. The upper histogram is the volume histogram and the lower, the hemoglobin concentration histogram. MCV and RDW are the mean and CV of the V-histogram, respectively. MCHC and HDW are the mean and SD of the HC-histogram, respectively.

RESULTS

Density-separated human red cells. MCV and MCHC values were obtained using the experimental light-scattering method and the aperture impedance technique for eight subpopulations of normal red cells with narrowly defined ranges of cell density. The data from one experiment are shown in Table 1. In the light-scattering method, both MCV and MCHC were independently measured, while in the aperture impedance method, MCV was measured and MCHC was calculated from the measured value of MCV. Since an increase in red cell density is the result of a decrease in cell water content and a consequent increase in cell hemoglobin concentration, the MCHC of red cells should increase with increasing cell density. MCHC, as measured by the light-scattering method, showed the expected monotonic increase with cell density. In contrast, MCHC, measured by the aperture impedance method, increased with cell density only for the first four density fractions. No relationship between cell density and MCHC was found for red cells isolated from the high-density regions of the Stractan gradient, and in fact, measured MCHC values began to decrease as cell density increased. Because the hemoglobin content (MCH) of isolated density subpopulations of red cells was essentially constant, for MCHC to increase with cell density it was necessary for the cell volume to decrease. Our light-scattering method was able to document both a

Table 1. Values of MCV and MCHC for Density-fractionated Normal Red Cells

	Experimental Light-Scattering Method		Aperture Impedance Method (Coulter Principle)	
Stractan Density Interface	MCV (fL)	MCHC (g/dL)	MCV (fL)	MCHC (g/dL)
<1.0785	106.5	27.6	109	27.8
1.0785–1.0829	98.6	29.8	97	31.2
1.0829–1.088	94.7	31.0	93	32.6
1.088–1.0919	91.4	32.1	91	33.3
1.0919–1.0964	84.3	34.6	93	32.5
1.0964–1.1009	77.0	37.0	92	32.9
1.1009–1.1056	70.6	40.1	100	30.3
1.1055–1.1102	69.9	41.1	101	30.0

decrease in MCV and an increase in MCHC with cell density. The difference in MCV and MCHC for red cells from the lowest and the highest density fraction were measured to be 36.6 fL and 13.5 g/dL by the light-scattering method, 18 fL and 5.5 g/dL by the aperture impedance method, and 38 fL and 14 g/dL by the manual method. Similar results were obtained in three other experiments using density-fractionated normal red cells.

The light-scattering measurement system also determines the HDW and the RDW. The HDW values for various subpopulations of density-fractionated red cells was measured to be between 1.1 and 1.3, whereas for whole blood from which these fractions were derived, it was 2.5. The smaller values for HDW of isolated subpopulations are to be expected, since the basis of cell separation is cell density; isolation of cells with a narrowly defined range of cell densities should result in narrow distribution of cell hemoglobin concentration as well. Interestingly, the RDW values for isolated subpopulations of red cells was not significantly different from that of whole blood (RDW of fractions 13.0 to 14.0 and of whole blood 15.0).

MCV and MCHC values for ten subpopulations of density-fractionated sickle red cells were also measured using the light-scattering and aperture impedance techniques. The data from one such experiment are shown in Table 2. As with normal red cells, MCHC of sickle red cells as measured by the light-scattering method increased monotonically with increasing density, whereas MCV progressively decreased with increasing density. However, the MCHC of the highest density fraction of sickle cells was not notably different from that of cells in the density fraction above it, probably because of our inability to induce complete sphering of dehydrated irreversibly sickled cells found in this density fraction. As with normal cells, MCHC values for sickle cells, as measured by the Coulter instrument, did not increase in parallel with cell density. Only a marginal increase in MCHC was evident, along with a marginal decrease in MCV with increasing cell density. Similar results were obtained in two other experiments using density-fractionated sickle cells.

Hydrated and dehydrated normal human red cells. The water content of hydrated and dehydrated red cells prepared using the nystatin procedure ranged from 60% to 140% of normal, with a corresponding MCHC range of 26 to 45 g/dL. Independent confirmation of the altered water content of nystatin-treated cells was obtained from demonstration of the expected changes in osmotic fragility and cell density. The values of MCHC obtained with the light-scattering and the aperture impedance methods agreed with those derived from the manual method in the range of 27 to 35 g/dL (Fig 4). However, at higher MCHC values, agreement existed only between the values derived by the manual method and those derived using the light-scattering method. As seen for the density-separated cells, the aperture impedance method markedly underestimated the MCHC values of the dehydrated cells (Fig 4).

Table 2. Values of MCV and MCHC for Density-fractionated Sickle Red Cells

Stractan Density Interface	Experimental Light-Scattering Method MCV (fL)	Experimental Light-Scattering Method MCHC (g/dL)	Aperture Impedance Method (Coulter Principle) MCV (fL)	Aperture Impedance Method (Coulter Principle) MCHC (g/dL)
<1.0785	98.2	25.4	95	31.5
1.0785-1.0829	95.4	30.0	93	32.2
1.0829-1.088	92.9	31.2	92	32.5
1.088-1.0919	90.4	33.6	94	31.8
1.0919-1.0964	86.8	35.5	91	32.8
1.0964-1.1009	76.6	38.2	89	33.6
1.1009-1.1056	75.1	39.2	83	36.0
1.1056-1.1102	74.0	40.6	86	34.8
1.1102-1.1149	74.5	41.7	83	36.0
>1.1149	77.1	41.8	85	35.2

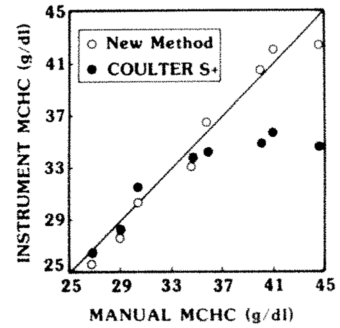

Fig 4. Relationship between MCHC derived by the light-scattering and electronic impedance methods *v* the spun-hematocrit method for hydrated and dehydrated human red cells. If all methods gave identical values for MCHC, the points would lie along the solid line of identity. Note that the data derived by the light-scattering method closely followed the line of identity over the whole range. In contrast, the electronic impedance-derived values were linear only between 27 and 35 g/dL and had a flat response above 35 g/dL.

The light-scattering system determines the volume and hemoglobin concentration of individual red cells independently. Consequently, it should be able to distinguish clearly between subpopulations of red cells with different volumes and hemoglobin concentrations. To test this premise, we measured the volume and hemoglobin concentration distributions of a mixture of hydrated and dehydrated red cells. The data are shown in Fig 5. The hemoglobin concentration distribution histogram for the mixture clearly shows two populations of red cells with different hemoglobin concentrations. In contrast, the two cell populations are less well separated in the volume distribution histogram.

Density-separated mammalian red cells. In order to determine whether the experimental light-scattering method could accurately measure the hemoglobin concentration of red cells with substantially smaller volumes than those of normal and modified human red cells, we measured MCHC of red cells from rabbit and sheep. The MCVs of red cells from these animals are much smaller than those of human red cells. Three subpopulations of human, rabbit, and sheep red cells with narrowly defined range of cell density were obtained using Stractan density gradients. Their cell indices as measured by the light-scattering method are shown in Table 3. The measured MCHC of red cells with the same range of cell density from all three species was found to be the same despite large differences in measured MCVs.

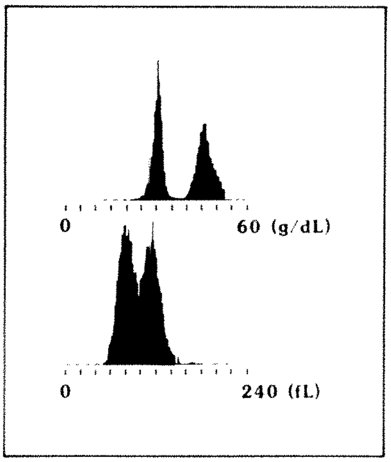

Fig 5. Volume and hemoglobin concentration distribution histograms for an equal mixture of hydrated and dehydrated human red cells, as determined by the light-scattering method. The MCV and MCHC values for the hydrated cells were 120.6 fL and 29 g/dL, respectively, while for dehydrated cells they were 78.9 fL and 42.1 g/dL, respectively.

Moreover, in all three species, the measured MCHC increased with increase in cell density, while MCV decreased with increasing cell density. Because red cells of identical cell density should have the same MCHC independent of cell volume, these data imply that the light-scattering method is indeed able to measure accurately the hemoglobin concentration of red cells with a wide range of cell volumes.

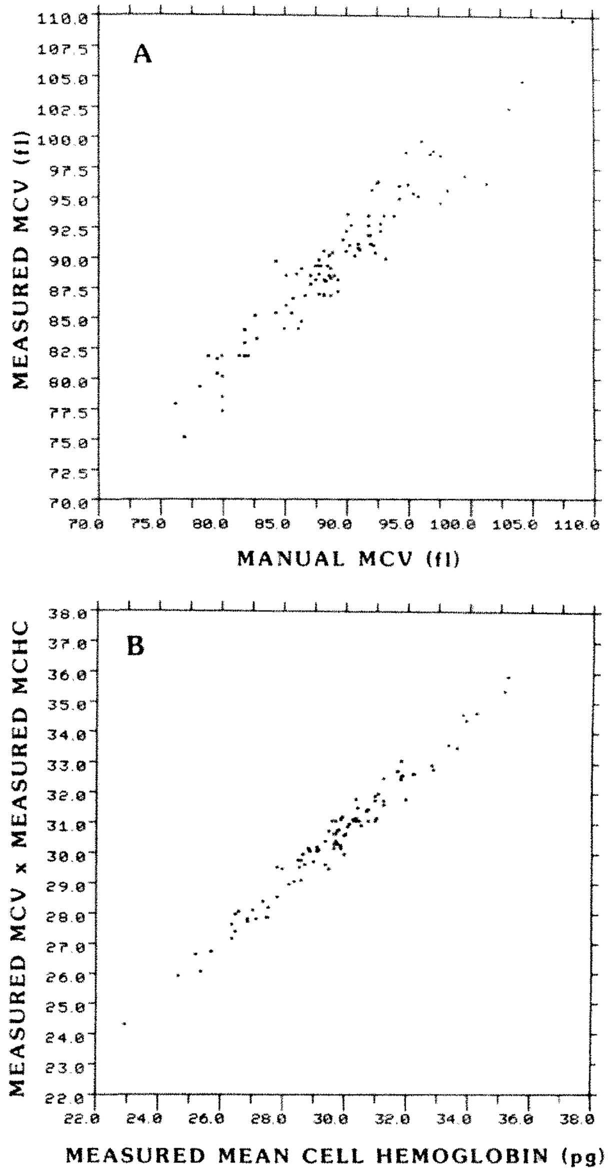

Fig 6. (A) Relationship between MCV measured by the light-scattering method and that derived from the manual method for 97 randomly selected hospitalized patients. The correlation coefficient obtained for these two MCV measurements is 0.96. (B) Relationship between two independently derived values of MCH. The MCH value shown on the abscissa was derived as the ratio of measured hemoglobin to red cell count, while the MCH value shown on the ordinate was obtained as the product of measured MCV and MCHC values from the light-scattering data. The correlation coefficient between the calculated MCH and measured MCH is 0.98.

Clinical blood samples. Red cell indices of whole blood obtained from 97 randomly selected hospitalized patients were also measured using our light-scattering method, the aperture impedance method, and the manual method. Figure 6A shows that MCV measured by the present method agrees well with the value obtained by the manual method. MCV of red cells ranged from 77 to 107 fL for these clinical samples. Figure 7A shows that for the same samples, MCHC measured by the light-scattering method showed reasonable agreement with the MCHC measured by the manual method. Although the correlation between the two MCHC values was not as good as that seen for MCV values, it was significantly better than that found between MCHC measured by the aperture impedance method and the manually derived MCHC (Fig 7B). We believe that the light-scattering method accurately measures MCHC, and that an even better correlation between measured MCHC and manual MCHC would result if we could improve the accuracy and precision of the manual method. This hypothesis is supported by our finding that the independently derived value of MCH showed an excellent correlation with the MCH calculated from measured values of MCV and MCHC (Fig 6B). Because MCH is derived from two parameters that can be accurately measured—hemoglobin and red cell count—our finding that this independently derived value of MCH and MCH calculated from measured values of MCV and MCHC (MCH = MCV × MCHC) were in excellent agreement implies that both cell volume and hemoglobin concentration

Table 3. Values of MCV and MCHC for Density-fractionated Red Cells From Different Mammalian Species

Stractan Density Interface	MCV			MCHC		
	Human	Rabbit	Sheep	Human	Rabbit	Sheep
1.088-1.0919	87.4	62.9	32.5	32.2	31.8	32.4
1.0919-1.0964	83.6	58.9	31.2	34.3	34.4	34.0
1.0964-1.1009	78.5	56.6	29.2	36.8	36.6	35.9

Values were determined by the experimental light-scattering method.

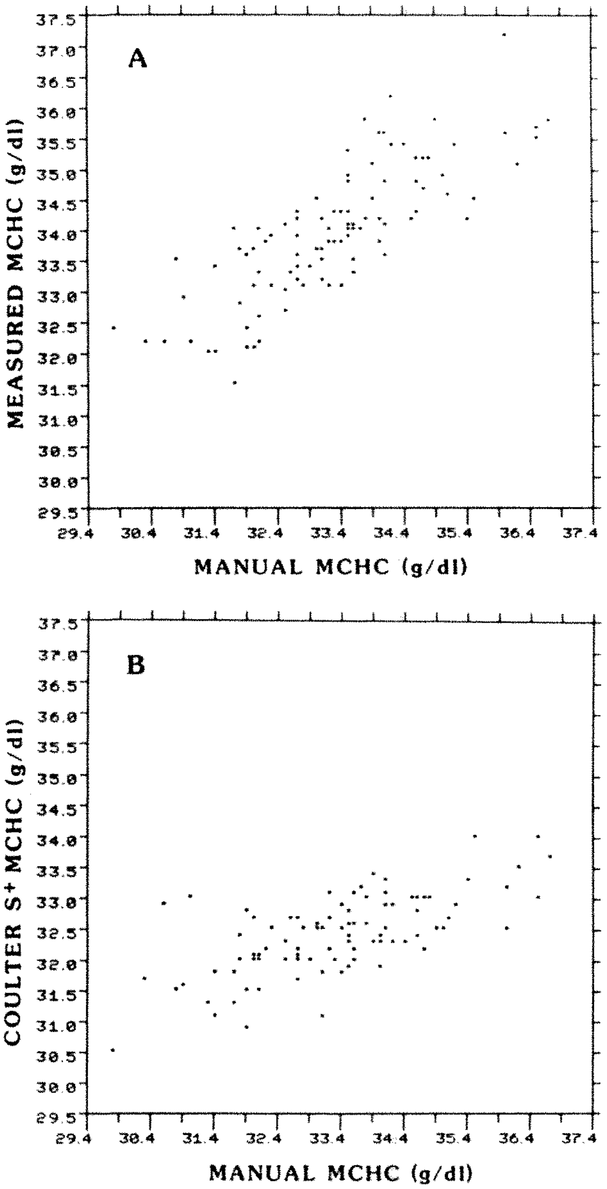

Fig 7. Relationship between MCHC measured by the automated method and that derived from the manual method for 97 randomly selected hospitalized patients. (A) Light-scattering method *v* manual method. (B) Aperture impedance method *v* manual method. The correlation coefficient obtained for MCHC measured by the light-scattering method and the manual method is 0.80, while for MCHC measured by the aperture impedance method and the manual method it is 0.67.

are indeed accurately measured by our light-scattering method.

Besides accurately measuring MCV and MCHC, the experimental light-scattering method also provided information on the widths of volume and hemoglobin concentration distribution. Because increased heterogeneity in red cell volume and hemoglobin concentration distributions have been previously documented in sickle cell disease,[11-13] we measured these parameters for sickle red cells from a small group of six patients, using the light-scattering method. The data are shown in Table 4. It can be seen that in five patients, the RDW values were increased, and in one patient, it was normal. In contrast, HDW was increased in all six patients. Analysis of the Stractan density distribution of normal red cells and sickle red cells from patients 2, 4, and 6 confirmed a direct correlation between increasing HDW values, increased broadening of cell density distribution, and hence hemoglobin concentration distribution. Examination of RDW and HDW values even in this small number of sickle cell patients clearly shows that these two parameters can vary independently.

Table 4. Cell Volume and Hemoglobin Concentration Parameters for Red Cells From Individuals With Sickle Cell Anemia

	Volume		Hemoglobin Concentration	
	MCV (fL)	RDW	MCHC (g/dL)	HDW
1	80.6	15.4	32.3	3.1
2	99.4	20.2	32.5	3.0
3	74.7	12.6	32.8	3.7
4	96.8	17.6	35.4	4.0
5	94.4	17.1	31.1	4.0
6	85.5	21.5	34.2	4.3
Normal range	80-94	11.5-14.5	33-36	2.4-2.9

A composite of various combinations of cell volume and hemoglobin concentration histograms seen in a randomly

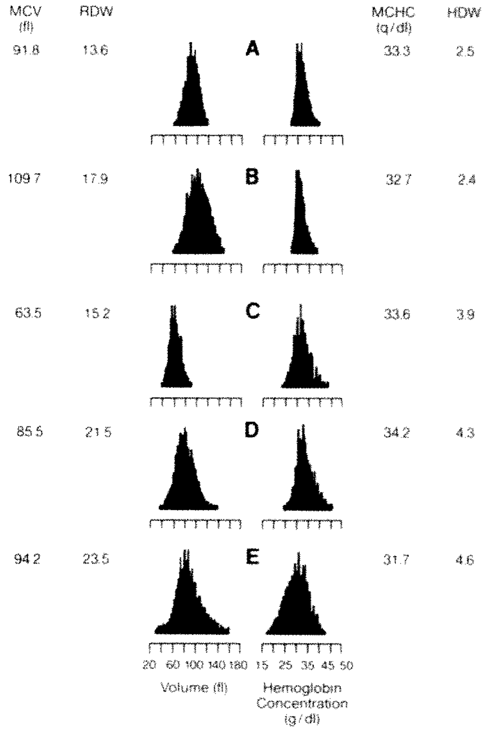

Fig 8. A composite diagram of various cell volume and hemoglobin concentration distribution histograms observed in randomly selected hospitalized patients. (A) Cells with a normal volume distribution and hemoglobin concentration distribution. (B) Cells with a broad volume distribution but a normal hemoglobin concentration distribution. (C) Cells with a normal volume distribution but a broad hemoglobin concentration distribution. (D) and (E) Cells with a broad volume and a broad hemoglobin concentration distribution.

selected set of clinical samples is shown in Fig 8. It can be seen that it is possible to have increased RDW with either normal or increased HDW as well as increased HDW with normal RDW. From this limited data, it is difficult to determine the relevance of the measured changes in RDW and HDW to either the underlying red cell dysfunction or the pathophysiology of anemia.

DISCUSSION

Data obtained during this study have enabled us to clearly establish that the experimental light-scattering method accurately measures both volume and hemoglobin concentration of individual red cells. Measurements derived from the experimental light-scattering method were accurate over a wide range of MCV (30 to 120 fL) and MCHC (27 to 45 g/dL) values. This is in contrast to currently available methods, in which MCHCs greater than 35 g/dL cannot be accurately measured[8] as a result of inaccurate MCV measurement of dehydrated cells. MCHC is a difficult parameter to ascertain even by the manual method, since variations in trapped plasma render hematocrit measurements used for deriving MCHC value inaccurate over a wide range of cellular hydration and deformability characteristics. Because of this, the accuracy of MCHC value derived by the manual method and its relevance have been questioned by a number of investigators.[24-28] The present method provides an independent and accurate measure of this important red cell index without being subjected to artifacts encountered in both the manual method and the currently used flow cytometric methods.

The one instance in which the experimental light-scattering method encountered a problem was in measuring the MCV and MCHC of the densest fraction of sickle cells. Because of the increased rigidity of irreversibly sickled cells found in this fraction, it was not possible to sphere these cells completely. As a result, our light-scattering theory algorithm could not accurately quantitate these parameters.[29] However, even for this sample, the values obtained with the light-scattering method were far more accurate than those obtained by the aperture impedance technique.

In the experimental light-scattering method, the influences of cell shape and deformability on volume and hemoglobin concentration measurements were mitigated by isovolumetric sphering and fixing of red cells. The concept of accurately obtaining volume measurement after isovolumetric sphering is not new; it was first proposed and used by Ponder in the 1930s and 1940s.[30] Mild fixation of cells after sphering allowed us to further eliminate the influence of cell shape changes that occur as a result of cell deformation induced by fluid forces generated during flow through the flow cell. Use of sphered red cells allowed us to apply the result of light-scattering theory quantitatively to our measurements and derive values for both particle volume and refractive index (hemoblogin concentration).

The ability to measure volume and hemoglobin concentration of individual red cells independently in turn allowed us to generate histograms of both volume and hemoglobin concentration distributions and from these derive the coefficient of variation for these distributions. A number of investigators have suggested that the RDW in conjunction with MCV may be used to discriminate among different red cell disorders.[31-35] Our results indicate that the volume measurements with the light-scattering technique are accurate over a larger range of cell volumes. Consequently, we anticipate that our RDW measurements will also be more accurate than those obtained with current instruments. Most important, the experimental method enables the quantitation of HDW. At present, the only way to derive such a value is by determining the distribution of red cell densities on analytical density gradients, a difficult and time-consuming procedure.[11-13,36,37] Elevated values for HDW have been documented for sickle cells, using density gradients, by a number of investigators.[11-14,36] Preliminary results obtained during the present study show that our method is capable of documenting this increase of HDW in sickle cell disease. In addition to increased HDW in sickle cell disease, it has recently been shown that dense dehydrated sickle cells are selectively removed from circulation during sickling crisis.[37] Moreover, increased HDW values have been reported for cord blood samples, β°-thalassemia red cells, and spherocytic red cells.[38] The availability of a rapid, quantitative method for determining HDW values should enable better evaluation of the role of the state of red cell hydration in sickle cell disease and other red cell disorders.

We also found during the present study that volume and hemoglobin concentration distributions can vary independently of each other in pathologic red cell samples. Application of our light-scattering method to a large number of clinical blood samples may enable one to define the usefulness of RDW and HDW parameters in the classification of various red cell disorders, as well as to define the role of cell volume and hemoglobin concentration heterogeneity in pathophysiology of red cell disorders.

A recent controversy has arisen concerning the clinical usefulness of the MCHC index, since it shows little variation in large patient population when determined by automated instruments.[22,24,25] The constancy of electronically measured MCHC values reported for patient populations may in part be explained by the inaccurate measurement of MCV of pathologic red cells as a result of their altered shape and deformability properties. The ability of our system to measure the MCHC of red cells independently and accurately may allow for objective evaluation of the biological variation of this parameter in various red cell disorders.

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Shortened Platelet Half-life in Multiple Myeloma

By Elke Fritz, Heinz Ludwig, Werner Scheithauer, and Helmuth Sinzinger

Various defects in platelet function have been reported as being associated with multiple myeloma. In 30 myeloma patients and 15 healthy controls, we investigated platelet survival using in vitro labeling of autologous platelets with ^{111}indium-oxine and measuring the in vivo kinetics of the radioisotope. Significantly shortened platelet half-life in patients averaged 73 hours, while platelet half-life in the healthy controls averaged 107 hours. In myeloma patients, serum levels of thromboxane B_2, β-thromboglobulin, and platelet factor 4 were significantly elevated; aggregation indices were within the pathological range; platelet counts and spleen–liver indices, however, were comparable to those of the healthy control group. No statistical correlation was found between platelet half-life and paraprotein concentrations. Our findings suggest an initial—so far unexplained—intravascular process of platelet activation and consumption that finally manifests in shortened platelet half-life. It seems that overt thrombocytopenia develops only when the compensatory capacity of the bone marrow finally becomes exhausted. Further studies should be able to elucidate the pathophysiologic processes involved.

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THROMBOCYTOPENIA is frequently observed in multiple myeloma, although it is seldom severe enough to cause overt bleeding.[1] In our own case material,[2] we found platelet counts below 10^{11}/L in 13% of newly diagnosed patients (n = 100); this ratio rose to 40% in terminal-stage patients (n = 50). Apart from quantitative deviations, pathological findings of platelet function have also been reported: impaired adhesiveness[1,3-5]; and adenosine diphosphate- or collagen-induced aggregation of platelets[3,5]; reduced platelet factor 3 release[3,6]; prolonged bleeding time[1,4]; impaired or delayed clot retraction,[3] and fibrin polymerization.[7,8] Abnormalities in platelet metabolism[9] have also been observed. In certain studies, the degree of functional impairment correlated with the amount of paraprotein[1,3,7,10] or of total protein[5] present.

Shortened platelet half-life has been reported in certain pathological conditions, including neoplastic growth of solid tumors, as well as in hematological malignancies.[11,12] Because our review of the literature indicated that platelet half-life had so far not been investigated in multiple myeloma patients, we determined to evaluate this parameter. We also attempted to confirm or to refute previous reports of increased amounts of circulating reversible platelet aggregates in multiple myeloma[13] determined by the aggregation index according to Wu and Hoak.[14] Both main pathways of platelet elimination were tested: we calculated the spleen–liver index as indicator of degradation in the reticuloendothelial system (RES), and we quantified (among other parameters of platelet activation) the plasma levels of β-thromboglobulin, a proposed indicator of intravascular platelet consumption.[15,16]

MATERIALS AND METHODS

Patients. Included in this investigation were 30 patients with multiple myeloma (15 male, 15 female) and 15 healthy controls (nine male, six female). Informed consent was obtained from all subjects. Median age was 62.6 years in the patient group and 62.0 years in the age-matched control subjects. Diagnosis of multiple myeloma was based on the presence of two out of the three following findings: (1) serum or urinary M-component, (2) bone marrow plasma cell infiltration >15%, (3) osteolytic bone lesions. Twenty-one patients presented with IgG, seven with IgA, and two with light-chain myeloma. The light-chain distribution of paraprotein showed 25 cases with kappa and five with lambda light chains. Examinations included ten patients at the time of diagnosis, nine within the first year of the disease, and 11 patients in whom multiple myeloma had been of long standing. In case of treatment, cytostatic or steroid drugs had been administered at least four weeks before testing, and neither patients nor controls had taken aspirin, indomethacin, or related compounds for at least one week.

Methods. To evaluate platelet half-life, the method of Hawker et al[17] was used: 16 mL of blood were collected without stasis; 4 mL of 0.15 mol/L acid citrate dextrose[18] were added simultaneously as anticoagulant. Platelet-rich plasma was obtained by sedimentation of the erythrocyte fraction for ten minutes at 22 °C and centrifugation of the supernatant at 150 g for five minutes. Centrifugation at 500 g for an additional ten minutes yielded the platelet pellet, which was suspended in 1 mL of Tyrode buffer, pH 6.2,[17] while the supernatant autologous plasma was saved. Labeling was performed with 100 μCi ^{111}indium-oxine (SGAE, Seibersdorf, Austria), a gamma radiation-emitting isotope that was selected because of its short half-life of 2.83 days and its reduced burden of radiation exposure as compared with ^{51}Cr.[19] The labeled platelet suspension was incubated for five minutes at 37 °C. After the inverted test tube was thoroughly washed and platelet pellet rinsed with warm buffer, the labeled platelets were resuspended in autologous plasma and reinjected into the patient's cubital vein. Radioactivity recovered in whole blood one hour after reinjection was 72% ± 6% and served as the 100% reference point. At least six additional samples of whole blood were collected in EDTA-containing test tubes (Greiner, Nürtingen, FRG) at precisely recorded intervals after injection of labeled platelets. The intervals approximated as closely as possible 3, 6, 9 or 12, 24, 30 or 36, 48, 54 or 60, 72 or 84, and 96 or 120 hours. All samples were simultaneously evaluated for residual radioactivity in a gamma counter (LKB Wallace, Helsinki). The resulting data were tested for linearity, and the optimal regression estimates, derived from a multiple-hit gamma function model, were fitted by the method of least squares, using SAS[20] on an IBM 43 41 computer (Institute of Medical Computer Science, University of Vienna). Only in cases of short platelet half-life was a nonlinear function—due to the fraction of residual free radioactivity in the blood—noted at the tail of the curve. In these instances, the first appearance of a statistically significant change in the slope function—always occurring well below the half-time mark—was defined as the cutoff point.

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The spleen–liver index was determined 18 to 24 hours after reinjection of labeled autologous platelets, using computerized scintigraphy in anteroposterior view. After insertion of regions at interest, the counts registered in the splenic area were divided by the actual counts obtained over the liver to yield a dimensionless index.

The aggregation index according to Wu and Hoak, a proposed measure of reversible circulating microaggregates, was determined by the method described.[14] Plasma concentrations of β-thromboglobulin and platelet factor 4 were evaluated, using commercially available radioimmunoassay systems (Radiochemical Center, Amersham, UK, and Abbott, North Chicago, Ill). Plasmatic thromboxane B_2 was measured in unextracted plasma samples using the double-antibody technique.[21] Two percent EDTA ensured anticoagulation, and 1 mg/mL of acetylsalicylic acid served as a blocker of cyclo-oxygenase.

Clinical parameters determined by routine methods included total serum protein concentration as well as serum and urinary M-component, serum calcium and hemoglobin levels, and peripheral platelet count. Additional data checked for possible correlations with the parameters of platelet function were sex, age, duration of the disease, extent of osteolytic bone lesions (determined by x-ray evaluation and whole-body scintigraphy), and estimated tumor cell load calculated according to the formula by Durie and Salmon.[22] In 15 patients, bone marrow aspirations were performed at the time of testing.

Statistical evaluation. All data were normally distributed either inherently or after logarithmic transformation. Only results of testing plasma concentrations of thromboxane B_2 included semiquantitative values (<70 pg/mL) and were therefore processed as frequencies. Mean values were compared, using Student's t test; for linear correlations, Pearson's product-moment correlations were calculated. For coefficients resulting from intercorrelations involving multiple comparisons within a single set of data, the level of significance was corrected according to Bonferroni statistics. The semiquantitative data on thromboxane B_2 were compared, using a chi-square test, while correlations were evaluated from Pearson's contingency correlation coefficients.

Possible influence of renal function on relevant parameters was tested by selecting all patients with serum creatinine findings of ≥20 g/L and determining the magnitude of each data item in relation to the total patient population. Mean coefficients of variation ([X − mean]/SD) were calculated for normally distributed data, while for skewed distributions results were expressed as percentiles.

Because the sample of 15 test series from our 15 healthy subjects was relatively small, we compared the data on platelet half-life in these 15 healthy controls with age- and sex-matched values from a previous comparable study involving 106 controls.[23] No significant difference was detected. Computer-assisted stepwise multivariate regression analysis, cluster and discriminant analysis, and matrix operations necessary to generate the three-dimensional plot were carried out on the IBM 43 41 computer.[20]

RESULTS

Platelet half-life was significantly shorter ($P < 10^{-5}$) in multiple myeloma patients than in healthy controls (Table 1). Figure 1 shows the distribution of individual values in these two groups. Platelet half-life in the controls ranged from 95 to 122 hours, with a median of 106 hours. In only seven of the 30 myeloma patients (less than 25%) were the values of platelet half-life within the normal range. In the remaining 23 patients, distribution appears to be skewed to the left, indicating pathological decrease. Median platelet half-life of the total patient group is 75.5 hours, ranging from 30 to 110 hours.

In patients as well as in controls, serial evaluation of radioactivity in whole blood yielded linear functions with varying slopes. Ratios of variances accounted for by the regression model were sufficiently high (r^2 median, .937; range, .690 to .992) to justify interpolation at 50% of residual radioactivity. Figure 2 gives one example each of the two types of kinetics observed in the patient group. In one patient, platelet half-life was 103 hours; the plot corresponds to those obtained in the control group, and it may well be taken as an example of normal findings. Kinetics in the other patient with a platelet half-life of only 32 hours are obviously pathological. However, the only certain difference is manifested in the steeper slope of the function.

Table 1 makes evident that mean platelet half-life in the patient group is significantly reduced and amounts to only 68% of the mean in the control group, and that no significant differences between the patient and the control group were observed in platelet count and spleen–liver indices. The aggregation index in the control population approaches the expected normal index of 1, while its significant reduction in the patient group indicates the presence of reversible circulating platelet microaggregates. Values for platelet activation parameters were significantly higher in the patient group than in healthy controls.

In three patients, serum creatinine was elevated to 22, 24, and 99 g/L, respectively. Mean coefficients of variation and

Table 1. Parameters of Platelet Functions in Patients With Multiple Myeloma and Healthy Controls

	Patients (N = 30)	Controls (N = 15)	Significance
Mean and standard deviation			
Platelet half-life	72.96 ± 24.72 hr	107.33 ± 9.26 hr	$P < 10^{-5}$
Platelet count	253,650 ± 103,012/μL	224,600 ± 104,084/μL	NS
Spleen–liver index	1.50 ± 0.62	1.47 ± 0.26	NS
Aggregation index	0.68 ± 0.10	0.95 ± 0.04	$P < 10^{-9}$
β-Thromboglobulin	64.64 ± 18.45 ng/mL*	24.53 ± 5.94 ng/mL*	$P < 10^{-9}$
Platelet factor 4	26.12 ± 8.05 ng/mL*	10.47 ± 2.98 ng/mL*	$P < 10^{-8}$
Median and range			
Thromboxane B_2	80 (<70–156) pg/mL†	<70 (max: <70) pg/mL†	$P < .001$

NS, not significant.

*Platelet-poor plasma.

†Plasma.

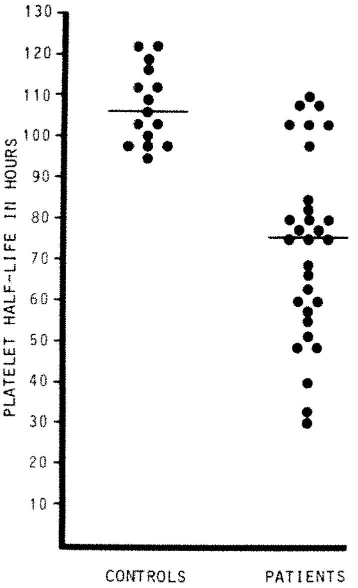

Fig 1. The median half-life of ^{111}indium oxine-labeled control platelets (n = 15) is 106 hours, while the median half-life of platelets from patients with multiple myeloma (n = 30) is significantly shortened to 75.5 hours ($P < 10^{-5}$).

percentiles for age, duration of the disease, total serum protein, serum M-component, hemoglobin, serum calcium, bone lesions, bone marrow plasma cell infiltration, tumor load, platelet count, platelet half-life, spleen-liver index, platelet aggregation index, thromboxane B_2, β-thromboglobulin, and platelet factor 4 showed no significant deviations in the two patients with 24 and 99 g/L of serum creatinine, respectively. In the third patient, with 22 g/L of serum creatinine, values for the duration of the disease, total serum protein, serum M-component, serum calcium, tumor load, and bone marrow plasma cell infiltration were significantly higher. His values for platelet half-life and platelet function (spleen-liver index, aggregation index, thromboxane B_2, β-thromboglobulin, and platelet factor 4), however, were within the 95% range of the patient group.

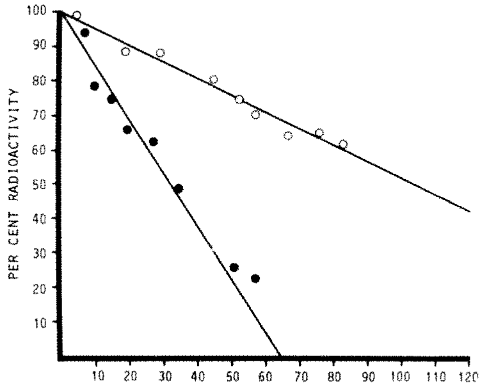

Fig 2. Kinetics of radioactivity in whole blood from two patients: Open circles represent a typical clearance function in cases with normal platelet half-life. Platelet half-life is 103 hours, the linear function is well fitted to the observations ($r^2 = .953$). The closed circles are derived from a patient whose platelet half-life is severely shortened to 32 hours. Again, the function is linear ($r^2 = .824$) with the possible exception of the lowest 25%.

All significant correlations are shown in Table 2. The pathological results of platelet function are strongly intercorrelated, but no correlation could be detected between platelet half-life and any of the clinical parameters investigated. High amounts of serum paraprotein were associated only with lower platelet count.

In the control group, intercorrelations were calculated between all available data (all parameters of platelet function, platelet count, sex, and age). Only β-thromboglobulin correlated significantly with platelet factor 4 ($r = .772$). If, by partial correlation, this shared proportion is eliminated in the patient group and the specific correlations of platelet half-life with β-thromboglobulin and with platelet factor 4 are calculated, the results are $r = -.424$, $P < .05$ and $r = -.463$, $P < .02$, respectively (data not shown).

By stepwise multivariate regression analysis, the suspected interdependence of the various parameters of platelet function could be confirmed: plasma concentration of thromboxane B_2 was sufficient to explain 72.6% of the total variance. By including β-thromboglobulin in the regression formula, the results could be improved and platelet survival could be predicted with high accuracy ($P < 10^{-10}$). All other parameters were eliminated by the procedure, indicating that—as to prediction of the independent variable half-life—they do not significantly add to the information derived from those two parameters. This interrelationship between thromboxane B_2, β-thromboglobulin, and platelet half-life is demonstrated in Fig 3. After smoothing of random errors of measurement, both dimensions of the regression plane are almost perfectly linear functions. The fit of the depicted model to the actual data is satisfactory ($r^2 = .866$).

Dividing the patients into two subgroups, (1) those diagnosed and not yet treated and (2) patients under treatment, and comparing the mean values of all variables, no significant differences were observed. Neither did stratification according to heavy-chain classes or light-chain types of paraprotein discriminate between the variables observed (data not shown).

Overt thrombocytopenia (platelet count of $<10^{11}$/L) was observed in 7% (two cases), while in 77% (23 patients), half-life fell below the range in healthy controls. To trace these interesting findings, we dichotomized the two variables with cutpoints at 90 hours of platelet half-life and at 10^{11}/L for platelet count. Patients were then assigned to one of three groupings: (A) platelet half-life as well as platelet count pathologically decreased; (B) platelet half-life abnormally shortened but platelet count normal (premature loss of platelets compensated for); or (C) platelet half-life as well as platelet count normal. The fourth possibility—normal half-life concurrent with thrombocytopenia—was not observed in our patient sample. The main results are shown in Table 3.

Being too small, subgroup (A) did not allow for the most interesting comparison between overt and compensated decimation of peripheral platelets. However, not only did subgroup (B)—compensated reduced platelet half-life—present significantly increased platelet activation when compared with (C), ie, patients with normal platelet half-life values,

Table 2. Correlations Observed in Patients With Multiple Myeloma

	T/2	β-TG	PF4	TxB_2	Serum M-Comp	Urinary M-Comp	Bone Lesions	Ca^{++}	Disease Duration
Platelet half-life		$r = -.772$ $P < .001$	$r = -.783$ $P < .001$	$r = -.852$ $P < .001$					
Platelet count					$r = -.426$ $P < .05$				
Spleen–liver index						$r = .666$ $P < .001$	$r = .391$ $P < .05$	$r = .408$ $P < .05$	
Aggregation index									$r = -.471$ $P < .02$
β-Thromboglobulin	$r = -.722$ $P < .001$		$r = .935$ $P < .001$	$r = .819$ $P < .001$			$r = .453$ $P < .02$		
Platelet factor 4	$r = -.783$ $P < .001$	$r = .935$ $P < .001$		$r = .823$ $P < .001$					
Thromboxane B_2	$r = -.852$ $P < .001$	$r = .819$ $P < .001$	$r = .823$ $P < .001$						

T/2, platelet half life; β-TG, β-thromboglobulin; PF4, platelet factor 4; TxB_2, thromboxane B_2.

but also the apparently "normal" subgroup (C) differed significantly from controls in regard to plasma β-thromboglobulin and platelet factor 4. Although platelet survival was shorter than normal in the two patients of subgroup (A), it was not in the shortest bracket. We did not state the highly significant result of comparing platelet half-lives in subgroups (B) and (C) because this outcome is logically trivial.

Multiple searches for possible patterns permitting identification of conditions favorable to or impeding the compensation of accelerated platelet loss remained inconclusive (data not shown).

Bone marrow plasma cell infiltration was determined by differential counts of bone marrow smears from 11 patients with platelet half-life <90 hours and from four patients whose 50% values of platelet clearance were within normal range. Median percentages of plasma cells found in the bone marrow were 29.5 for the first and 12.0 for the second subgroup. The values for all 15 patients ranged from 3% through 91%, the highest percentage determined in the only thrombocytopenic patient included in the sample. Bone marrow plasma cell infiltration correlated significantly with serum calcium, urine M-component, tumor mass per m^2 body surface, spleen–liver index, and duration of the disease (r between .646 and .784). The inverse correlation with peripheral platelet counts also exceeded the limit of significance ($r = .702$).

DISCUSSION

Theoretically, a pathological shortening of platelet half-life may be traced to one of two basic causes: to an intrinsic

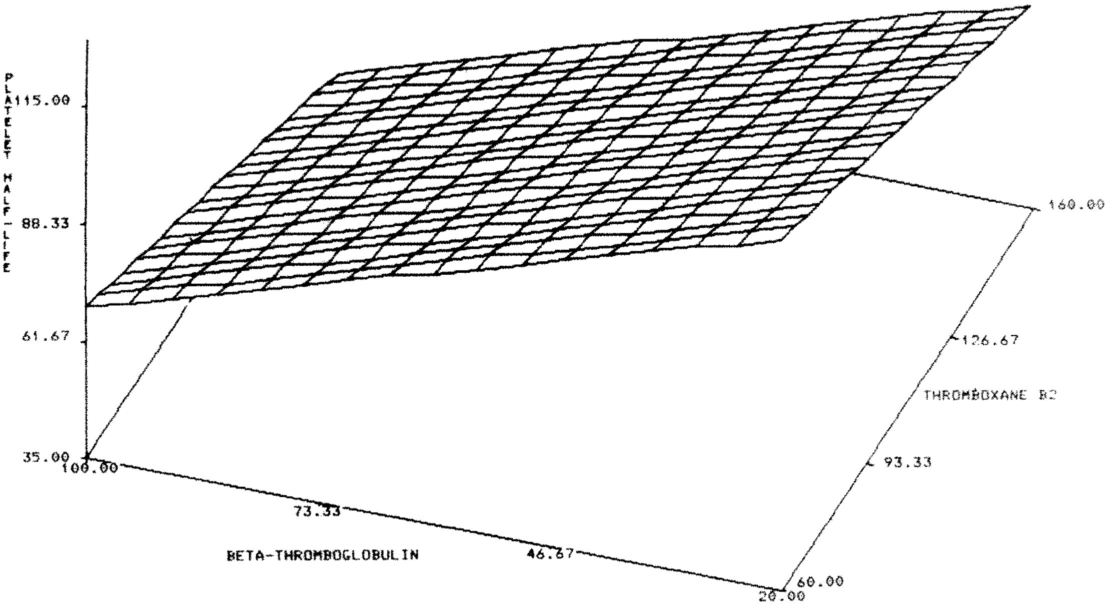

Fig 3. Interrelations between platelet half-life, thromboxane B_2, and β-thromboglobulin. Front: Low plasma concentrations of thromboxane B_2 and β-thromboglobulin are associated with normal platelet half-life. Rear: The lowest platelet half-lives are observed concomitantly with the highest plasma levels of thromboxane B_2 and β-thromboglobulin. The plane has been derived from the actual data after smoothing of random errors of measurement.

Table 3. Parameters of Platelet Functions After Grouping by Platelet Half-life and Platelet Count

	A	B		C		
Platelet half-life:	<90 hr	<90 hr	Significance	>90 hr	Significance	
Platelet count:	<100,000	>100,000	B *v* C	>100,000	C *v* Controls	Controls
Platelet half-life*	70.0–17.1	59.5–16.7		104.6–4.2	NS	107.3–9.3
Platelet count*	70,500–21,900	247,100–60,800	NS	309,900–139,200	NS	224,600–82,200
Thromboxane B_2†	93 (<70–116)	96 (<70–156)	$P < .01$	<70 (<70–80)	NS	<70 (<70)
β-Thromboglobulin*	60.5–12.8	68.4–16.0	$P < .001$	42.9–9.9	$P < .001$	24.5–5.9
Platelet factor 4*	24.5–3.9	29.4–7.2	$P < .001$	19.1–6.0	$P < .001$	10.5–3.0
N	2	21		7		15

NS, not significant.

*Mean – SD.

†Median (range).

defect[24] of the platelet, leading to premature destruction in the spleen or at other prominent sites of RES activity, or to an extrinsic defect, ie, environmental conditions effecting accelerated platelet loss. Extrinsic factors, in turn, may also operate by two fundamentally different mechanisms: by either rendering blood platelets more vulnerable to degradation or effecting an abnormally high rate of intravascular platelet consumption.[15,25] The significantly shortened platelet half-life observed in our myeloma patients cannot be attributed unequivocally to one or the other of these three possible causes.

Both intrinsic impairment due to a disease-associated deviant clone of megakaryocytes, which produces qualitatively inferior, or even defective, platelets, and influences resulting from the distinctly abnormal intravascular environment can be assumed to cause reduced platelet survival in multiple myeloma. Seeing the findings obtained in our study in the light of these hypotheses, it is challenging to weigh their value in providing possible explanations.

Although the absence of correlation between platelet half-life and paraprotein concentration appears to argue against environmental factors, the data collected on serial evaluation of radioactivity in the patients' blood fail to support the assumption of a fraction of pathological platelets being present among normal specimen. If two survival functions with distinctly different slopes were combined, the resulting hyperbolic function would not permit a satisfactory fit of a linear model to the observed data points. The linearity of the clearance function suggests a uniform population of platelets with fairly constant survival times.

When considering external factors that might exert adverse influences on platelet survival in multiple myeloma, chemotherapy constitutes an obvious possibility. However, platelet half-life in patients under treatment did not differ from that in patients not yet treated. The expected negative correlation between duration of the disease and half-life of platelet clearance also could not be confirmed. Neither cytostatic drugs nor possible consequences of steroid treatment appear to be responsible for the shortening of platelet survival in multiple myeloma.

In previous reports on abnormal platelet function in patients with plasma cell dyscrasias, the observed defects were mostly attributed to paraprotein coating of the platelets,[1,3,5,7,10] which may enhance their tendency to form microaggregates or render them more susceptible to destruction. Although our patient collective exhibited highly significant reductions in the aggregation indices (ie, increased amounts of reversible circulating microaggregates), we were unable to confirm the proposed correlation between the amount of serum or urine paraprotein and parameters of platelet function. Nor did stratification into subgroups according to heavy-chain class or light-chain type of the paraprotein molecule yield any significant results. No association of any observed feature of platelet pathology with IgG,[10] IgA,[1] or lambda light chain[8] could be detected.

These findings, however, do not necessarily rule out all influence of the plasma M-component on the platelet population. Even though the total amount of paraprotein seems to be of little relevance as such, detrimental effects from immunoglobulin aggregates, polymers, or free light chains have to be considered. Unlike most of the previous studies,[1,4,6,13] we excluded cases of Waldenström's macroglobulinemia, and the number of our patients with light-chain myeloma was too small to permit drawing specific conclusions in this regard. In this connection it is interesting to note that—in contrast to bone marrow plasma cell infiltration—paraprotein concentration did not correlate with the duration of the disease. As myeloma progresses, the tumor cells, to some extent, lose their capacity to secrete paraprotein, and deviant antibody molecules become more frequent.[2,26] Possibly it is the quality rather than the quantity of paraprotein that determines the effect it has on the platelet population.

The spleen–liver indices in our patient group did not differ from those observed in healthy controls; there were no indications of extraordinary sequestration or degradation of platelets. Unexpected was the observation that the parameters of platelet activation were significantly increased, even in patients whose platelets still showed normal life spans. On the other hand, in no patient was reduced platelet half-life found concurrently with normal values in all parameters of platelet activation. We ruled out the possibility that the elevated serum levels of platelet factors were due to diminished renal clearance in patients with impaired kidney function.

Our findings do not support the assumption that in multiple myeloma, premature elimination of platelets is due to mere coating or cross-linking of inert platelets, ie, of platelets not activated and not exhibiting release reaction. It rather appears that initially some intravascular process[15,16] activating the platelets takes place. Our data suggest that the extent of such a proposed platelet activation has to exceed a certain threshold before it manifests as a measurable shortening of platelet half-life.

A second threshold, in the bone marrow's capacity to compensate for increased platelet consumption, appears to be indicated by the fact that in more than 75% of our patients, platelet half-life fell below the normal range, while only less than 10% showed overt thrombocytopenia. Unfortunately, we had not included bone marrow examinations in our investigational plan as a general feature, but in 15 patients—among them one of the two patients with thrombocytopenia—this procedure was performed concurrently with the testing of platelet function. Although the small number of cases does not allow for definitive conclusions, the results merit consideration on theoretical grounds: in general, shortened platelet half-life appears to be associated with larger numbers of myeloma cells in the bone marrow, but there is no necessity to presuppose a linear correlation. Our findings showing that platelet half-life is not correlated with paraprotein concentration permit the following conclusion: if more plasma cells secrete larger amounts of paraprotein, this fact alone does not constitute a greater hazard to the platelet population. However, the significant correlation between high percentage of bone marrow tumor cell infiltration and low platelet count suggests that the progression of the disease may play a key role in the capacity of the bone marrow to provide adequate amounts of platelets in compensation for their premature peripheral elimination.

Even though the magnitude of platelet activation findings depends on the test being used, our interpretations rely on differences between patients and controls observed under identical conditions. The method of determining microaggregates, as proposed by Wu and Hoak, is prone to produce artifacts due to the passive participation of formaldehyde-fixed platelets in aggregation[27] so that the indications of platelet aggregation need to be confirmed by independent investigators.

We tentatively propose a model explaining the processes involved in the reduction of platelet half-life and the eventual manifestation of thrombocytopenia in myeloma patients: very early in the course of the disease, some intravascular stimulus appears to activate the platelet population; the extent of the ensuing consumption and possibly also of increased extravascular destruction may be reflected in platelet survival time, leading from a slight or moderate to a pronounced shortening of platelet half-life. There seems to exist, however, the capacity to compensate for this diminution of the platelet pool to a large extent and for relatively long periods. For the continuous platelet loss to become manifest as overt thrombocytopenia, apparently a second event or process must take place: severe bone marrow depression as a consequence of massive infiltration of the bone marrow by myeloma cells.

Future investigations are needed to determine possible agents of intravascular platelet activation, and they should include differential counts of bone marrow smears. The hypothesis of a primarily normal platelet population being affected by environmental influences could be tested by labeling experiments using allogeneic platelets. Should the observed interrelationship between platelet half-life and plasma concentrations of thromboxane B_2 and β-thromboglobulin be confirmed, simple screening assays included in clinical routine examinations may be sufficient to monitor the risk of thrombocytopenia. It would be of particular interest to include in future studies all thrombocytopenic patients without overt bleeding complications, in order to determine the conditions necessary to compensate for the platelet loss. If the processes involved in the platelet pathophysiology associated with multiple myeloma can be more fully elucidated, more efficient treatment—or even prevention—of overt thrombocytopenia in multiple myeloma may become possible.

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Anomalous Clustering of Underglycosylated Band 3 in Erythrocytes and Their Precursor Cells in Congenital Dyserythropoietic Anemia Type II

By Michiko N. Fukuda, George Klier, John Yu, and Paolo Scartezzini

Congenital dyserythropoietic anemia type II (CDA II or HEMPAS) is a genetic anemia caused by membrane abnormality. Our previous studies indicated that in HEMPAS, erythrocytes band 3 and band 4.5 are not glycosylated by polylactosaminoglycans. The present study was aimed at determining how such underglycosylated band 3 behaves in erythrocyte membranes. By using anti-band 3 antibodies, immunogold electron microscopy revealed that band 3s are clustered in HEMPAS erythrocyte membranes. By freeze-fracture electron microscopy, band 3s were also seen as lightly clumped intramembrane particles on a protoplasmic fracture face. Erythrocyte precursor cells stained by anti-band 3 antibodies showed that band 3s are present in the cytoplasmic area of the reticulocytes as scattered single particles. However, in young erythrocytes in which intracellular membranes are almost degenerated, band 3s were clustered in the cytoplasmic area of the cell. These observations suggest that band 3s cluster before they are incorporated into the plasma membranes of HEMPAS erythrocytes. In contrast to band 3, glycophorin A detected by anti-glycophorin A antibodies did not show a noticeable difference between normal and HEMPAS. Such a clustering of band 3 may cause abnormal localization of band 3-associated proteins and may thus result in the macroscopic membrane abnormality seen in HEMPAS erythrocytes.

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LACTOSAMINOGLYCAN PROTEINS are a group of glycoproteins present in plasma membranes. Their carbohydrates often serve as cell surface antigens, including Ii, ABH, and SSEA-1, which change drastically during cellular differentiation, embryonal development, and oncogenesis.[1-3] Although structural and immunochemical studies on lactosaminoglycans have progressed in recent years,[4] the functional aspect of these carbohydrates has not yet been described, except for the involvement of fucosylated lactosaminoglycans in cellular recognition in early embryogenesis of mice.[5,6] If the carbohydrate moiety of lactosaminoglycan proteins has some role, a lack of such carbohydrates should result in the disturbance of normal cellular functions.

Congenital dyserythropoietic anemias (CDA) are genetic erythropathies accompanied by bone marrow erythroid hyperplasia and erythroblastic polynuclearity.[7] In all types of CDAs, membrane abnormalities are prominent features on electron microscopic examination.[8] CDA type II are also called HEMPAS (*h*ereditary *e*rythroblastic *m*ultinuclearity associated with a *p*ositive *a*cidified *s*erum test) because the erythrocytes are lysed by acidified serum, which is 30% of the normal population.[9] Anselstetter et al[10] first noticed that band 3 glycoproteins in HEMPAS erythrocytes migrate faster than those of normal on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Since then, the lower molecular weight (mol wt) of band 3 has been recognized as a specific molecular feature of this disease.[11-16] A proteolytic fragmentation study indicates no deletion of peptide from HEMPAS band 3.[12] However, decreased glycosylation of band 3 and band 4.5 was demonstrated by cell surface labeling[14,15] and chemical analysis of isolated HEMPAS band 3.[13] Thus, in HEMPAS, these proteins are not glycosylated by polylactosaminyl carbohydrates. Instead, these carbohydrates are accumulated as polylactosaminyl lipids.[15] Therefore, a blockage of the glycosylation pathway to form lactosaminoglycan proteins may result in a shift of glycosylation to the lipid acceptor.

Although the primary genetic defect of HEMPAS is unknown, the decreased glycosylation of band 3 and band 4.5 is specific to HEMPAS erythrocytes.[14,15] The present study was aimed at determining how such underglycosylated band 3s behave in erythrocyte membranes and at clarifying mechanism of the membrane abnormality seen in HEMPAS. We report here anomalous clustering of band 3 in HEMPAS erythrocytes and their precursor cells as revealed by electron microscopy.

MATERIALS AND METHODS

Cells. Peripheral blood samples obtained from four unrelated HEMPAS patients were subjected to analyses by immunogold staining and freeze-fracture electron microscopy. Blood samples from HEMPAS patients, cases 1, 2, and 3, were collected at Genova, Italy. At each occasion, blood samples from healthy individuals were also obtained. All blood samples were sent to San Diego with coolant. Accordingly, these blood samples had been kept at 4 °C for three to four days before being subjected to immunoelectron microscopy or freeze-fracture electron microscopy. HEMPAS case 4[17] blood sample was collected by Dr R.C. Card, at the University of Saskatchewan, Canada, and was immediately sent to San Diego. Therefore, the case 4 HEMPAS blood had been kept at 4 °C for 24 hours before it was subjected to analysis. At approximately the same time, normal blood was collected at San Diego, stored at 4 °C until HEMPAS case 4 sample arrived, and used for control. Sickle cell anemia patient blood that had a high reticulocyte count (14%) and bone marrow aspirates from a healthy individual were kindly provided by Dr A.P. Varki, Cancer Center, University of California, San Diego. Normal blood samples that were fresh, stored at 4 °C for three to four days, or stored at 4 °C for more than 3 weeks (outdated blood) were also examined to verify the results obtained.

Antisera. Sheep anti-band 3 serum[18] was kindly provided by Dr M. Fukuda, La Jolla Cancer Research Foundation. Rabbit antiglycophorin CB-2 serum[19] was kindly provided by Dr H. Furthmayr, Yale University School of Medicine, New Haven, Conn. Rabbit antigoat IgG and goat antirabbit IgG were purchased from Cappel,

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Cochranville, Pa. Anti-I (Ma) serum and anti-i (Dench) serum were kindly donated by Dr E. Giblett, Puget Sound Blood Bank, Seattle, and Dr M.C. Crookston, Toronto General Hospital, respectively.

Immunoelectron microscopy. The procedure described by Tokumitsu et al[20] for fixation and immunostaining was modified. Briefly, blood cells were fixed at room temperature in freshly prepared PLP fixative (10 mmol/L $NaIO_4$, 75 mmol/L lysine, 37.5 mmol/L sodium phosphate buffer, 2% paraformaldehyde, pH 6.2)[21] for 15 minutes. The cells were washed three times in 0.1 mol/L sodium phosphate buffer, pH 7.4, containing 1 mg/mL bovine serum albumin and 0.02% saponin (Sigma Chemical Co, St Louis) for one hour. The cells were incubated overnight with or without (control) anti-band 3 (1:200) or antiglycophorin A (1:200) antibodies diluted with phosphate buffer containing saponin at 4 °C. After washing three times with phosphate buffer containing saponin, the cells were incubated with a second antibody complexed to colloidal gold[22] for one hour at room temperature. The cells were washed three times with phosphate buffer containing saponin and twice with phosphate buffer. Finally, the cells were treated with glutaraldehyde and processed for electron microscopy.[23] A Hitachi K-600 electron microscope was used.

Freeze-fracture electron microscopy. Blood samples for freeze-fracture electron microscopy were washed three times with phosphate-buffered saline. After adding a mixture with 40% glycerol, the cells were frozen and prepared by standard techniques[24] at −110 °C in a Balzers apparatus using an electron bombardment to cast the replicas.

RESULTS

Distribution of band 3 in normal and HEMPAS erythrocytes. The peripheral blood from four unrelated HEMPAS patients was subjected to analysis. In all cases, erythrocytes showed increased agglutination titers with anti-I antibodies and showed positive agglutination with anti-i antibodies. On SDS-PAGE, all cases showed sharper and lowered mol wt of band 3. Cell surface labeling by galactose oxidase/$NaB[^3H]_4$ method showed decreased labeling on band 3 and band 4.5 and accumulation of HEMPAS-glycans (polylactosaminyl lipids), which is consistent with the results reported previously.[14,15]

Figure 1 shows immunogold staining of HEMPAS and normal erythrocytes by using anti-band 3 antibodies. In normal erythrocytes, band 3s were stained mostly as single or double gold particles, whereas in HEMPAS, band 3s were often shown as groups of gold particles. It appeared that almost all HEMPAS erythrocytes have a wide range of cluster sizes but not such a mixed cell population of normal (with single and small-sized clusters) and abnormal (with large-sized clusters) erythrocytes.

The distribution of gold particles among blood samples is shown in histograms (Fig 2). The data show that normal erythrocytes are stained mostly as single or small-sized clusters by anti-band 3 antibodies. Because band 3 is known to be present as dimer in erythrocyte membranes, we tentatively assumed single and double gold particles as normal band 3 distribution. The mean of such normally distributed band 3s was obtained as 71.9% ± 4.9% by the four experiments on normal blood (shown in Fig 2). Conversely, HEMPAS cases 1, 3, and 4 revealed great degrees of clustering patterns of band 3s. The mean of the normally distributed band 3s was obtained as 28.3% ± 1.1% by these three HEMPAS cases. As shown in Fig 2, HEMPAS case 2 showed a lesser degree of band 3 clustering. The value of the normally distributed band 3s for HEMPAS case 2 was 46.2%, which is significantly low compared with the mean value obtained by normal. These observations strongly suggest that band 3s are clustered in HEMPAS erythrocyte membranes.

In contrast to these results on band 3, the distribution of glycophorin A by using antiglycophorin A antibodies was indistinguishable between normal and HEMPAS erythrocytes. As shown in Fig 3, immunogold staining of glycophorin A gave a somewhat clustered or patched pattern in both normal and HEMPAS erythrocytes.

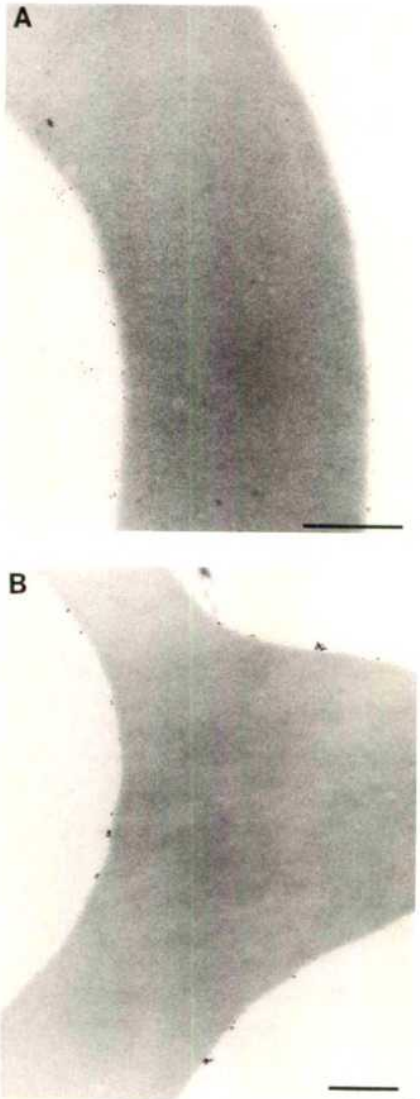

Fig 1. Immunogold staining of band 3. Normal (A) and HEMPAS patient (B) erythrocytes were treated with sheep anti-band 3 antibodies, followed by gold-coated rabbit antisheep IgG antibodies. Erythrocytes from Normal-1 and HEMPAS case 1 are shown. In the control experiment without sheep anti-band 3 antibodies, erythrocytes were not stained with gold particles (not shown). Note that band 3s are seen as clusters on HEMPAS erythrocyte membranes. Scale bars show 0.5 μm.

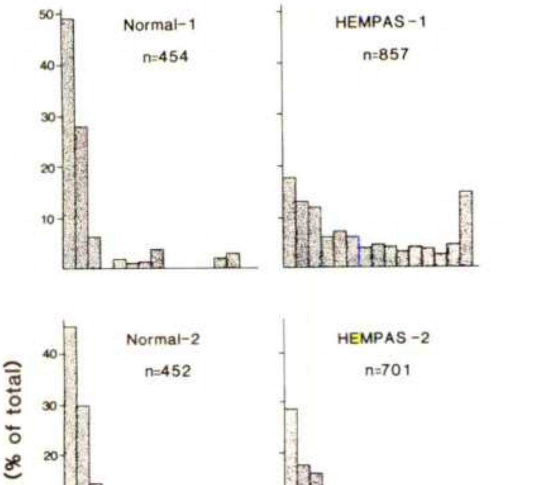

Fig 2. Histograms showing the extent of clustering gold particles for band 3 in normal and HEMPAS erythrocytes. The immunogold staining patterns obtained by four experiments are presented. Normal and HEMPAS blood samples were treated exactly the same way in each experiment and were subjected to immunogold electron microscopy.

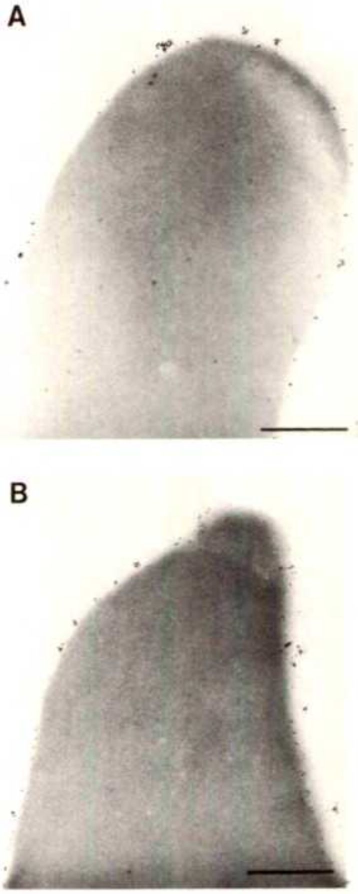

Fig 3. Immunogold staining of glycophorin A. Normal (A) and HEMPAS patient (B) erythrocytes were treated with rabbit anti-glycophorin A antibodies, followed by gold-coated goat antirabbit IgG antibodies. Erythrocytes from Normal-1 and HEMPAS case 1 are shown. Scale bars show 0.5 μm.

Freeze-fracture electron microscopy of HEMPAS erythrocytes. In order to further verify the results on band 3, normal and HEMPAS erythrocytes were examined by freeze-fracture electron microscopy. It is known that band 3s can be seen as intramembrane particles by freeze-fracture electron microscopy.[24,25] Figure 4 shows the images of intramembrane particles on a protoplasmic fracture face obtained by the freeze-fracture method. In HEMPAS, intramembrane particles clump together, leaving open spaces between the clusters. In normal erythrocytes, intramembrane particles are evenly spread. Furthermore, the isolated membranes from HEMPAS erythrocytes also demonstrate similar degrees of aggregation of intramembrane particles on examination with freeze-fracture electron microscopy (results not shown). Thus, the aggregation of intramembrane particles was apparently not induced by the cryoprotecting agents, such as glycerol, used in the preparations of intact cells for freeze-fracture studies.

Intracellular clustering of band 3 in erythrocyte precursor cells. Band 3s were also detected in erythrocyte precursor cells in the peripheral blood of HEMPAS. Figure 5 shows the immunogold staining for band 3 in two immature erythrocytes. Band 3s were detected as scattered single particles in the cytoplasmic area of the younger cell (on the left in Fig 5), which has intracellular membranes with a light deposition of hemoglobin. Another cell, shown in Fig 5 (on the right), is most likely a young erythrocyte, since the inside area of this cell is darkened by hemoglobin deposition but is not darkened as evenly as a mature erythrocyte (see Figs 1 and 3). Intracellular membrane structures appear to be degenerated in this cell. Band 3s were detected on the cell surface as well as in the cytoplasmic areas of this cell. It is noteworthy that almost all the band 3s were detected as clustered gold particles in such young erythrocytes in HEMPAS.

Figure 6 shows a high magnification of band 3 cluster present in the cytoplasm of a HEMPAS reticulocyte. No

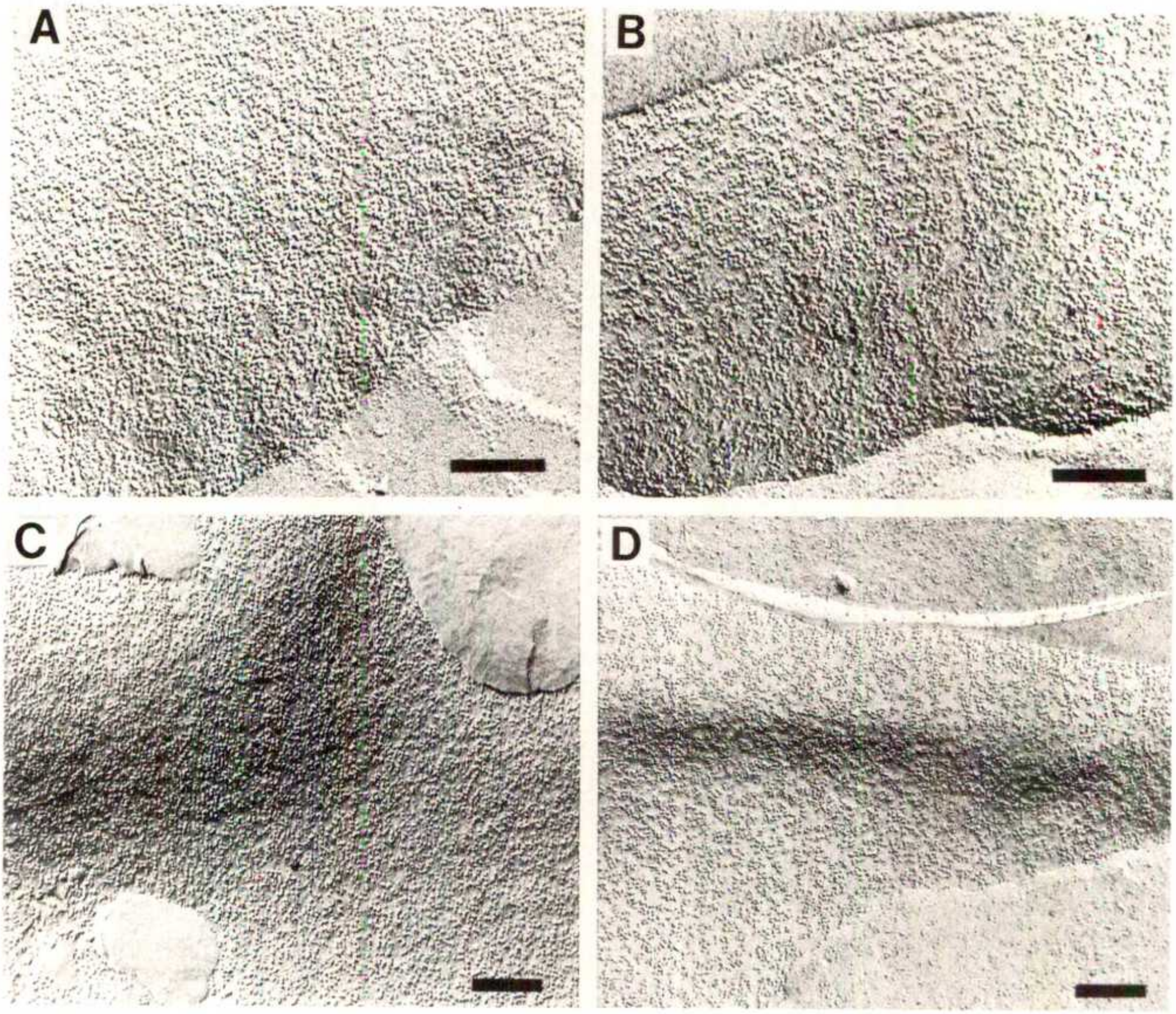

Fig 4. Freeze-fracture electron micrography of normal and HEMPAS erythrocytes. Intramembrane particles on a protoplasmic fracture face are shown: A, normal-2; B, HEMPAS case 2; C, normal-1; D, HEMPAS case 1. HEMPAS and normal cells were processed exactly the same way in each experiment, including storage periods. Each set of experiments, ab or cd, was done on two occasions. Scale bars show 0.2 μm.

particular membrane structure or organella were associated with the cluster.

In HEMPAS case 1 blood samples, we found a total of 19 cells that appeared to be at the stage of reticulocytes or young erythrocytes similar to the cells shown in Fig 5. All of the 19 cells were stained by anti-band 3 in the cytoplasmic area, and among them, eight cells were found to have intracellular clustering of gold particles, as shown in Fig 5 (the right cell) and in Fig 6. In HEMPAS cases 2, 3, and 4, the numbers of immature cells we found were 1, 4, and 1, respectively. The cytoplasmic area of these cells also stained by anti-band 3 antibodies, and one of them, from HEMPAS case 3, showed a staining pattern similar to that of Fig 5 (the right cell). In order to evaluate these results, we examined reticulocytes present in the normal bone marrow as well as in the peripheral blood of sickle cell anemia patients. As reported previously, band 3 of sickle cell's erythrocytes are glycosylated normally by lactosaminoglycans.[15] We observed more than 20 reticulocytes in each control experiment. Although all of them were stained by anti-band 3 in cytoplasmic area, and some of them were stained on cell surface as well, none of the normal reticulocytes or the sickle cell's reticulocytes showed the intracellular clustering of band 3s. Note that in these experiments, we did not perform any additional treatment on cells, such as density centrifugation. We have just found immature erythrocytes in a large number of sections of immunogold electron microscopy. This is because centrifugation in stractan solution[26] performed for reticulocyte enrichment apparently damaged erythrocyte membranes, and clustered immunostaining pattern of band 3 was induced in normal erythrocyte membranes. We therefore decided to look for reticulocytes without any treatment for enrichment.

Although it is not clear whether the electron-dense masses

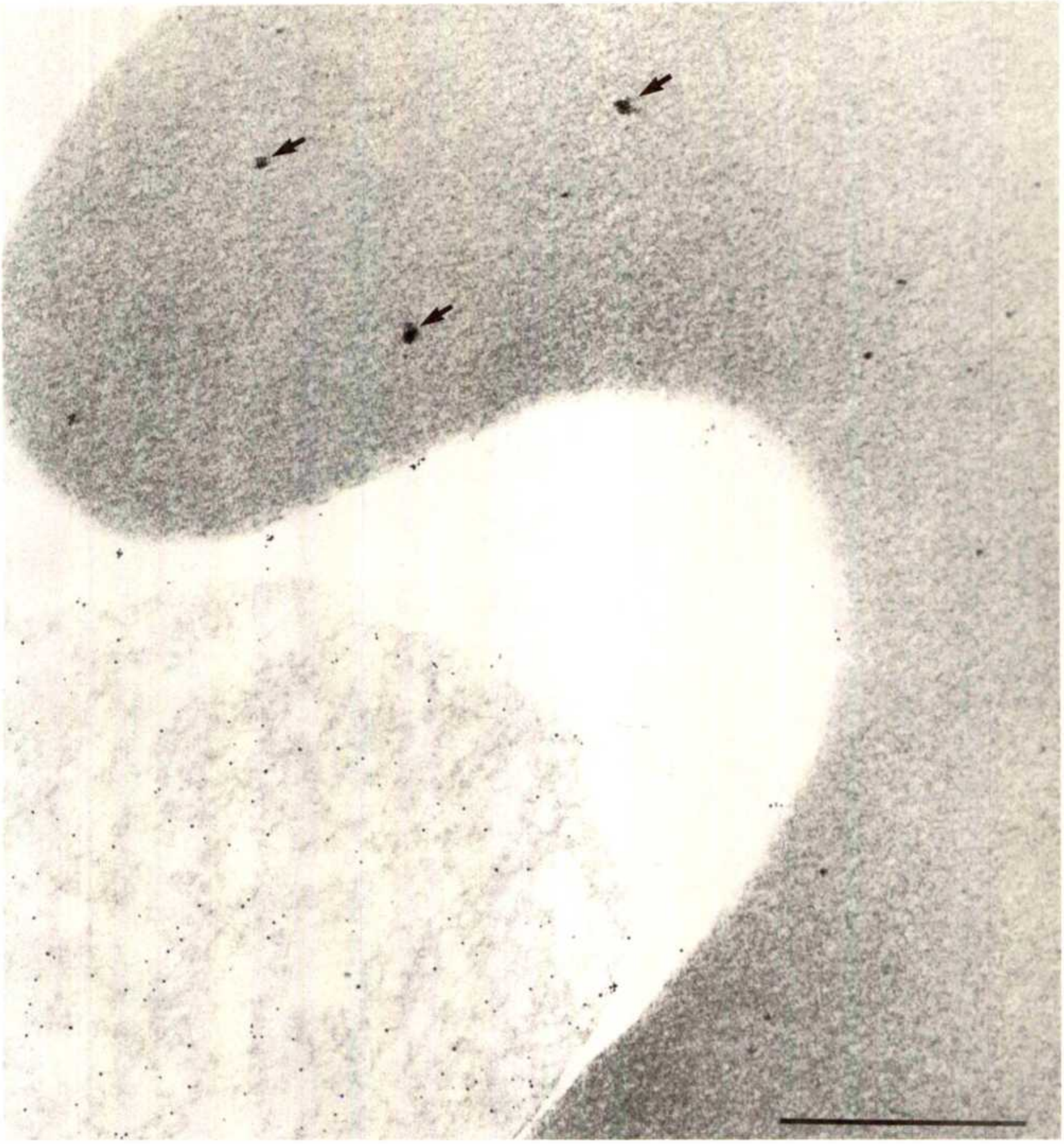

Fig 5. Immunogold staining of band 3 in HEMPAS reticulocytes. Reticulocytes (lower left) and young erythrocyte (right) are shown. Band 3s are distributed all over on the intracellular membrane structures and are not clustered in reticulocytes (lower left). In the young erythrocyte or matured reticulocyte (right), intracellular membranes disappeared and intracellular space is darkened because of the accumulation of hemoglobin. Cells from HEMPAS case 1 are shown. Clusters of band 3s are shown by arrows. Scale bar shows 0.5 μm.

(as seen in Fig 6) are made solely by gold particles or contain other components, such as ferritin, the involvement of band 3 molecules in such clusters is strongly suggested by the control experiments: (1) HEMPAS cells that were processed in the same manner but without anti-band 3 antibodies showed no electron-dense mass in the cytoplasmic area of reticulocytes. (2) Immature HEMPAS erythrocytes that were observed in immunogold staining by anti-glycophorin A antibodies showed a scattered single particle staining pattern in the cytoplasmic area but did not show intracellular clustering of gold particles (see later).

The localization of glycophorin A in this stage of reticulocyte is shown in Fig 7. Significant amounts of glycophorin As are already present on the cell surfaces of reticulocytes,

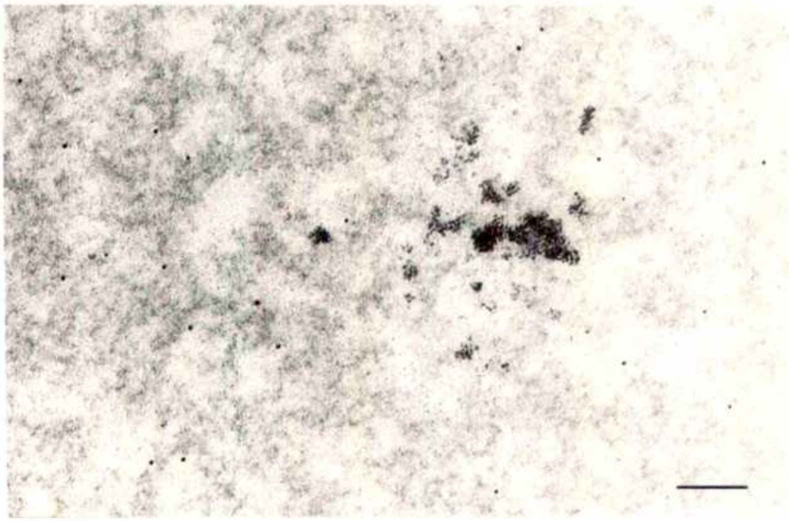

Fig 6. Immunogold staining of band 3 in HEMPAS reticulocytes. Band 3s are seen as a large mass as well as scattered single particles. No particular membrane structure or organella are seen near the band 3 clusters. Cells from HEMPAS case 1 are shown. Scale bar shows 0.1 μm.

which is in contrast to that of band 3, where they are present mostly in cytoplasm (see Fig 5). The result of Fig 7 is consistent with the previous report that expression of glycophorin takes place earlier than that of band 3.[27] Glycophorin As were also detected in the intracellular area as scattered single particles. We observed a total of 12 immature erythrocytes (nine cells in HEMPAS case 1 and three cells in HEMPAS case 3), which are at the similar maturation stage as shown in Fig 7. Although all of these cells were stained antiglycophorin A antibodies, both in intracellular area and cell surface, none of them had intracellular clustering of gold particles.

These results indicate (a) that band 3s are actively synthesized in reticulocytes and distributed evenly in the cytoplasm, probably in the endoplasmic reticulum; (b) that band 3s are aggregated together as the reticulocyte matures and the intracellular membrane system degenerates; and (c) that band 3s are finally localized on the plasma membrane as clusters in HEMPAS erythrocytes. A schematic model for band 3 clustering is presented in Fig 8.

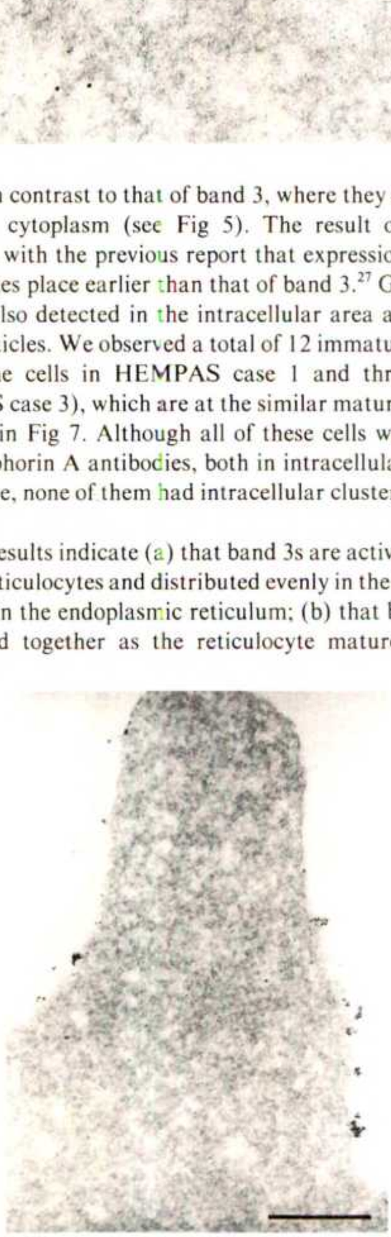

Fig 7. Immunogold staining for glycophorin A. A matured HEMPAS reticulocyte is shown. Glycophorin As are seen on the cell surface as well as inside the cell. No intracellular clustering of glycophorin A was detected. A cell from HEMPAS case 1 is shown. Scale bar shows 0.5 μm.

DISCUSSION

Band 3, the major intrinsic protein of erythrocyte membranes, constitutes up to 30% of the total membrane proteins.[28,29] Two types of carbohydrate chains are attached to

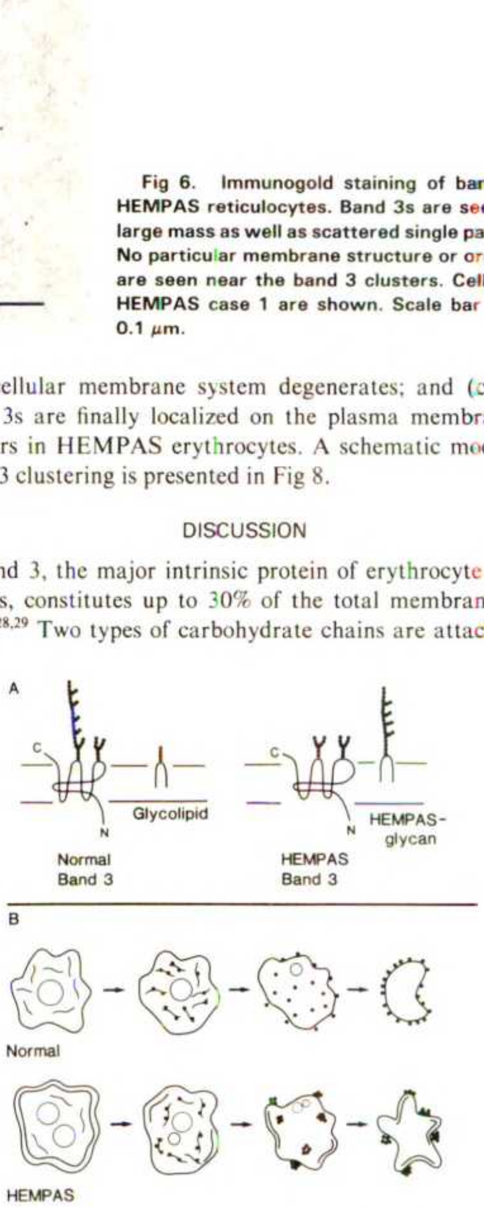

Fig 8. The schematic model for band 3 clustering in HEMPAS erythroid cells. (A) Status of polylactosamines in normal and HEMPAS erythrocytes. In normal, band 3s are glycosylated by lactosaminyl repeats. In HEMPAS, glycosylation of band 3 is incomplete and polylactosaminyl lipids (HEMPAS glycans) are accumulated. (B) In normal, band 3s are synthesized in reticulocytes and moved to plasma membranes. Band 3s are evenly distributed on erythrocyte membranes. However, in HEMPAS, band 3s cluster before they move to the plasma membrane. As a result, the clustered distribution of band 3s is seen in HEMPAS erythrocyte membranes. Such abnormal behavior of HEMPAS band 3 may be caused by the lack of a large carbohydrate moiety (as shown in A) and the subsequent increase of hydrophobicity of this protein.

the band 3 peptide[30,31]: the so-called lactosaminoglycan (mol wt 8,000) and the complex-type carbohydrate (mol wt 2,000). The side chains of lactosaminoglycan are made of galactose and *N*-acetylglucosamine repeats, and these outer side chains and their modified forms can express antigens, such as Ii- and ABH-antigens.[4] Previous studies on HEMPAS erythrocytes indicated that band 3s are not glycosylated by lactosaminyl repeats, which explains the lowered mol wt of HEMPAS band 3.[15] Band 3 purified from HEMPAS erythrocytes had predominantly short carbohydrate chains (M.N. Fukuda, A. Dell and P. Scartezzini, unpublished data).

The immunogold staining experiment and freeze-fracture electron microscopy led us to conclude that band 3s are clustered in plasma membranes of HEMPAS erythrocytes (Figs 1, 2, and 4). In these experiments, we used HEMPAS erythrocytes that had been stored at 4 °C for 24 hours (case 4) or for three to four days (cases 1, 2, and 3). Because normal blood was processed along with HEMPAS blood, including storage periods, the clustered distribution of HEMPAS band 3 must be reflecting characteristic status of HEMPAS band 3s, ie, HEMPAS band 3s cluster in situ or are ready to cluster in plasma membranes. Previously established knowledge[25] on normal erythrocytes and our experiment (data not shown) using outdated blood have indicated that clustering of band 3s would not be induced over a prolonged (longer than 3 weeks) storage at 4 °C.

The clustering of HEMPAS band 3 was also demonstrated in immature erythrocytes (Figs 5 and 6). These observations suggest that the clustering process takes place inside the cells at the time that intracellular membrane systems disappear. The primary factor for the leading clusters of band 3 could be the lack of a large carbohydrate moiety and the subsequent increase of total hydrophobicity of this protein. It is known that the peptide moiety of band 3 is strongly hydrophobic in its composition and its behavior in aqueous solution.[28] The disappearance of intracellular membrane systems may allow band 3s to move instead of being fixed in membranes, and thus may result in the clustering of band 3.

When normal erythrocytes are treated with endo-β-galactosidase, carbohydrates on band 3 and band 4.5 are removed with a concomitant loss of Ii-antigens from erythrocytes.[32] However, the endo-β-galactosidase treatment of erythrocytes did not induce clustering of band 3 (G. Klier and M.N. Fukuda, unpublished observations). Therefore, the removal of carbohydrates from band 3, which is already embedded in the plasma membrane, is not effective enough to cause clustering. Consistently, the proteolysis of intact erythrocytes and the removal of the extracellular domain of band 3 do not induce redistribution of band 3.[25] This evidence agrees with the present observation shown that band 3s cluster intracellularly before they are incorporated into the plasma membranes.

We believe that the images obtained by freeze-fracture electron microscopy (Fig 4) closely represent the real distribution of band 3s. The immunogold staining method seems to be much more sensitive to detecting the clustered band 3s. This is probably because clustered molecules are easily recognized by antibodies for antigenic multivalencies. Therefore, we should take into account that the results obtained by immunostaining (Figs 1 and 2) possibly exaggerate the magnitude of the clustering.

Recently, the band 3 clustering caused by hemoglobin denaturation followed by hemichrome binding was shown.[33] We cannot exclude such a possibility of involvement of hemoglobin for mechanism of HEMPAS band 3 clustering. However, one should also be reminded that band 3 clustering has not been reported in any genetic anemia, whereas clustering of band 3s can be easily recognized by freeze-fracture electron microscopy.[24,25] Altered globin synthesis is observed in both CDA type I and CDA type II (HEMPAS).[16] However, freeze-fracture electron microscopy on CDA type I cells did not reveal noticeable anomalies in plasma membranes.[34] No alteration of band 3 in terms of the mol wt has been found in CDA type I.[10,16] From this evidence, we cannot strongly correlate hemoglobin breakdown as the cause of band 3 clustering in HEMPAS erythrocytes.

The cytoplasmic domain of band 3 provides binding sites for several other proteins (see references 28 and 29 for reviews). Therefore, the clustering of band 3 may result in abnormal localization of associated proteins as well. For example, the association of the membrane skeleton proteins[35] should be considered as one of the factors for the irregularly contracted shape of HEMPAS erythrocytes.[9] It is likely that the clustering of band 3s results in the irregular network of membrane skeletons. If so, such erythrocytes would easily be deformed.

In contrast to band 3, another erythrocyte glycoprotein, glycophorin A, did not show any noticeable difference between normal and HEMPAS on immunoelectron microscopy (see Fig 3). In the previous study by cell surface labeling, glycophorins did not show significant anomalies specific to HEMPAS in terms of glycosylation.[15] Glycophorin A, the major molecular species of erythrocyte sialoglycoproteins, is composed of as much as 60% carbohydrates.[36] Most of the carbohydrates are the so-called *O*-glycosidic chains, each of which is short and sialylated.[37] No polylactosaminyl chains attach to glycophorin A in normal erythrocytes. The present result, therefore, shows that anomalies in HEMPAS specifically occur in lactosaminoglycan proteins, but not in all glycoproteins.

During the differentiation of erythroid cells, the expression of polylactosaminoglycans is greatly increased at the erythroblast stage.[27,38] Vainchenker et al[39] showed that the membrane abnormality of HEMPAS is seen in erythroblastic cells but not in uncommitted cells. In erythroblasts, lactosaminoglycans are carried by proteins that have an apparent mol wt of 90 to 110 kd.[27] Therefore, it is possible that these glycoprotein(s) in HEMPAS erythroblasts are also clustered due to the insufficient glycosylation, and thus cannot perform their normal functions. Further study should address the distribution of the lactosaminoglycan proteins in HEMPAS erythroblasts by using antibodies specific to these early-stage specific glycoproteins.

Although the primary genetic defect of HEMPAS has not yet been defined, various changes in the glycosylation of proteins and lipids have been noticed.[12-15,40,41] The lack of polylactosamine on proteins and the accumulation of polylactosaminyl lipids in HEMPAS erythrocytes suggest that the glycosylation pathway to glycoproteins is blocked.[15]

If such an anomaly is caused primarily by a failure of the glycosylation, then the membrane anomaly should be observed in other types of cells that normally express lactosaminoglycan proteins. Therefore, cases of HEMPAS in which the membrane abnormality was found in granulocytes, macrophages, platelets, and megakaryocytes[42,43] agree with this view, since granulocytes are now known to be rich in lactosaminoglycan proteins.[44] HEMPAS patients with widening of the diploe of the skull, with mental abnormalities, or with detachment of retinal pigmented epithelial cells have been reported.[42,45,46] Some of the abnormalities seen in these tissues may be caused by underglycosylation of membrane proteins. In severe cases of HEMPAS, these abnormalities become visible anomalies. Further studies on genetic disease HEMPAS will, therefore, provide us with understanding of the role of lactosaminoglycan proteins in blood cells as well as various cells in tissues.

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Monocytes Stimulate Fibroblastoid Bone Marrow Stromal Cells to Produce Multilineage Hematopoietic Growth Factors

By Virginia C. Broudy, Kenneth S. Zuckerman, Shobha Jetmalani, John H. Fitchen, and Grover C. Bagby, Jr

In previous studies we have found that monocytes produce soluble factors that stimulate human umbilical vein endothelial cells to produce granulocyte-macrophage colony-stimulating activity (CSA), burst-promoting activity (BPA), and megakaryocyte colony-stimulating activity (Meg-CSA) as well as factors that stimulate T lymphocytes and neonatal fibroblasts to produce CSA. To test the hypothesis that monocytes would similarly stimulate the production of hematopoietic growth factors by autologous bone marrow stromal cells, multiply-passaged adherent fibroblastoid cells derived from the bone marrow of normal volunteers were exposed to conditioned media prepared by incubating autologous peripheral blood monocytes in complete medium for three days. When conditioned media from stromal cells incubated in monocyte-conditioned medium were compared with those of stromal cells cultured in the absence of monocyte-conditioned medium, BPA was increased fourfold and CSA was increased more than 30-fold. We conclude that mononuclear phagocytes recruit stromal cells of the marrow to produce multilineage growth factors in vitro. We suggest that these monocyte-derived recruiting activities may play an important role in orchestration of hematopoietic growth factor production by cells of the marrow microenvironment.

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STUDIES IN THIS and other laboratories have indicated that mononuclear phagocytes constitutively produce a soluble factor (or factors) that stimulates T lymphocytes,[1] fibroblasts,[2] and endothelial cells[3-5] to produce granulocyte-macrophage colony-stimulating activity (CSA) in vitro. We have also found that endotoxin enhances the production of this granulopoietic monokine and that with or without endotoxin, monocytes are more efficient recruiters of CSA production than they are producers of CSA.[5] We have recently reported that monocytes also produce a factor that stimulates the production of burst-promoting activity (BPA) and megakaryocyte colony-stimulating activity (Meg-CSA) by endothelial cells.[6,7] Because T lymphocytes, fibroblasts, and endothelial cells are all known to be components of the hematopoietic microenvironment,[8,9] we have suggested that mononuclear phagocytes may play a pivotal regulatory role in hematopoiesis by recruiting cells of the microenvironment to produce multilineage hematopoietic growth factors.[6] In our previous studies, assays were performed using conditioned media from allogeneic cells, and the fibroblasts and endothelial cells used in those studies were of neonatal origin; umbilical veins were the source of endothelial cells and foreskin the source of fibroblasts. Accordingly, we sought to determine whether autologous monocytes could stimulate the production of hematopoietic growth factors by normal bone marrow fibroblastoid cells.

MATERIALS AND METHODS

Peripheral blood and bone marrow samples were obtained from informed and consenting healthy adult volunteers. These studies were approved by the institutional review boards at the institutions where the samples were collected. The overall design of the experiments was similar to those of our previous studies.[1-3,5,6] Specifically, we first cultured monocytes to generate monocyte-conditioned media (MCM), which contain the monokines. Second, we added the MCM to monolayer cultures of fibroblastoid marrow cells derived from bone marrow of the same volunteer whose monocytes were used to produce the MCM. Third, we carried out CSA and BPA assays on the MCM, fibroblastoid cell-conditioned medium (FCM), and the conditioned medium produced by fibroblastoid cells cultured in MCM (FCM$_M$).

Monocyte cultures. Monocytes were isolated from peripheral blood of healthy volunteers as previously described.[2,5] Low-density cells were obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) centrifugation.[10] Low-density cells (less than 1.077 g/mL) were incubated with sheep red blood cells, and E rosettes were removed by density centrifugation.[11] Monocytes were isolated from the E rosette-negative population by adherence to serum-coated dishes.[12] Monocytes thus isolated were 90% nonspecific esterase positive and 95% viable by trypan blue exclusion. The monocytes were cultured at a concentration of 1×10^5 cells per milliliter in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 2 mmol/L L-glutamine and 15% lactoferrin-depleted[13] fetal calf serum. After three days of incubation at 37 °C in an atmosphere of 7.5% CO_2 in air, the MCM was harvested and centrifuged to remove cell debris.

Marrow fibroblastoid stromal cells (MFSCs). MFSCs were isolated from bone marrow of healthy adults. Low-density marrow cells were obtained from single-cell suspensions of heparinized marrow aspirates using Ficoll-Hypaque as described. These cells were incubated in Medium 199 (GIBCO) supplemented with 20% fetal calf serum and 2 mmol/L L-glutamine. Cells were seeded at 5×10^6/mL in T flasks and were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium was changed twice weekly. MFSC cultures could be established from >90% of the normal donors we studied and usually reached confluence by 3 to 4 weeks. The cells were maintained for up to 6 months in culture. Primary outgrowths of cells at confluence were detached from the culture flasks with 0.02% EDTA-trypsin, washed, diluted 3:1, and replated.

The cells were characterized by cytochemical staining with acid phosphatase, alpha-napthylacetate esterase, alkaline phosphatase, periodic-acid Schiff (PAS), and myeloperoxidase by standard techniques. Surface antigenic characteristics of the cells were deter-

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mined by staining with the pan-T lymphocyte monoclonal antibodies T28 and S33 (a gift of Peter Beverley, Imperial Cancer Research Fund, London) and with the monocyte-specific monoclonal antibodies M1 and M120, followed by a fluorescein-labeled second antibody, and were then analyzed on a cytofluorograph (Ortho 50H). Fixed cells were also stained with rabbit antihuman factor VIII-related antigen, followed by a fluorescein-labeled second antibody, and were then examined by fluorescence microscopy. The cytochemical stains, surface and cytoplasmic antigen analysis, and functional studies (latex bead phagocytosis, nitroblue tetrazolium reduction) were each performed on two to three MFSC cultures.

CSA assay. Assays of conditioned media for CSA were performed according to previously published methods.[3] Briefly, 10^5 low-density, nonadherent, E rosette-depleted human marrow cells were cultured in 1 mL 0.9% methylcellulose in alpha medium supplemented with 15% fetal calf serum. The methylcellulose was layered on an agar base (0.5% wt/vol in McCoy's 5a medium) to which had been added 0.1 mL of the sample to be assayed. For dose–response experiments, 0.01 mL, 0.05 mL, or 0.10 mL of the conditioned media were added to the agar underlayer. Human placental-conditioned medium (HPCM), a potent source of CSA, served as a positive control. Colonies (aggregates greater than 39 cells) were counted after seven to 11 days of culture at 37 °C in a fully humidified atmosphere of 7.5% CO_2 in air.

BPA assay. Clonal growth of BFU-E derived from monocyte-depleted (adherence to serum-coated tissue culture dishes) peripheral blood mononuclear cells served as our BPA assay, as described previously.[6,14] One hundred thousand monocyte-depleted mononuclear cells were grown in 0.3 mL plasma clot cultures in the presence of 1 U/mL human urinary erythropoietin (National Heart, Lung, and Blood Institute). Samples being tested for BPA were added (10% vol/vol) to the plasma clots. For dose–response experiments, conditioned media were added at 1%, 5%, and 10% final concentration to the plasma clots. BFU-E (greater than 50 benzidine-positive cells) were counted after 14 days of culture at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Potentiator assay. MCM and FCM were mixed together (1:1), incubated for 24 hours at 37 °C, and assayed for CSA and BPA as described.

Inhibitor assay. To test for the presence of inhibitors of granulopoiesis, 0.1 mL of each conditioned medium was added to marrow cells cultured in methylcellulose containing HPCM as a source of CSA. Inhibitors of erythropoiesis were assayed by adding conditioned media (10% vol/vol) to peripheral blood low-density cells cultured in plasma clots containing phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) as a source of BPA. Colony growth in the presence of the conditioned media was compared with colony growth with HPCM or PHA-LCM alone.

Statistical analysis. We used the Kruskal-Wallis test with multiple range testing to compare colony growth under the various conditions described.

Table 1. Characteristics of Human Bone Marrow Fibroblastoid Stromal Cells

Cytochemical staining:	Acid phosphatase positive
	Alkaline phosphatase positive
	α-Napthylacetate esterase positive
	PAS positive
	Myeloperoxidase negative
Surface antigens:	T28 negative (T lymphocyte)
	S33 negative (T lymphocyte)
	M1 negative (monocyte)
	M120 negative (monocyte)
Cytoplasmic antigen:	Factor VIII antigen negative (endothelial)
Functional studies:	Nitroblue tetrazolium reduction negative
	Latex bead phagocytosis negative

RESULTS

Characteristics of stromal cells. MFSCs were propagated for as many as 16 subcultures. In our early studies, in which we had hoped to obtain an outgrowth of endothelial cells, we routinely added 250 μL/mL of endothelial cell growth factor.[15] It soon became clear that the stromal cells were not endothelial cells and that the cells grew quite well without the addition of this material. The phenotypic characteristics of these cells are outlined in Table 1. Morphologically, these cells appeared fibroblastoid and stained positively with acid phosphatase, alkaline phosphatase, alpha-naphthylacetate esterase, and PAS. They did not stain with myeloperoxidase. Cytofluorographic analysis revealed that the cells did not stain with T lymphocyte-specific monoclonal antibodies T28 and S33, nor with monocyte-specific monoclonal antibodies M1 and M120. The cells lacked factor VIII-related antigen, were nonphagocytic (latex beads), and did not reduce nitroblue tetrazolium. Scanning electron microscopy revealed spindle-shaped cells with long cytoplasmic projections. MFSCs did not support the proliferation of autologous CFU-GM in primary cultures or in cultures recharged with autologous low-density nonadherent bone marrow cells. Considering the homogeneous fibroblastoid nature of MFSCs, their cytochemical characteristics and surface phenotype, and taking into account our ability to culture them to confluence for more than 15 subcultures, we believe that these cells are most consistent with adventitial reticular cells originally described by Westen and Bainton[16] and are probably the same as the marrow fibroblast colony-forming cells described by other groups.[17-21]

Hematopoietic growth factor production by stromal cells. The data shown in Table 2 are derived from MFSC cultures established from six normal volunteers. Each stromal cell culture was incubated with and without MCM made from autologous monocytes. Unstimulated MFSCs did not constitutively produce significant amounts of CSA. The MCM, which contained only small amounts of CSA, stimulated a 30-fold increase in CSA production by the marrow stromal cells (Table 2). In some experiments, the CSA of

Table 2. Production of CSA and BPA by Marrow Fibroblastoid Stromal Cells

Conditioned Media Added	CFU-GM	BFU-E
0	0	1.0 ± 0.6
MCM	4 ± 2	3.7 ± 1.4
FCM	3 ± 3	4.1 ± 1.0
FCM_M	100 ± 20	16.3 ± 3.0

The data are expressed as the number of colonies per 10^5 cells (mean ± SEM) from six experiments. Conditioned media were tested for growth factor activity at a final concentration of 10%. FCM_M was significantly more stimulatory to both CFU-GM and BFU-E growth than was MCM or FCM ($P < .01$). MCM, monocyte-conditioned medium; FCM, marrow fibroblastoid stromal cell-conditioned medium; FCM_M, conditioned medium produced by fibroblastoid stromal cells cultured in MCM made from autologous monocytes.

Table 3. Results of Inhibitor Assays

Conditioned Media Added	CFU-GM (% control)	BFU-E (% control)
0	100	100
MCM	101 ± 13	89 ± 6
FCM	96 ± 11	88 ± 11
FCM$_{\mathrm{M}}$	138 ± 33	92 ± 10

MCM, FCM, and FCM$_{\mathrm{M}}$ were added at a final concentration of 10% to cultures containing either HPCM as a source of CSA or PHA-LCM as a source of BPA. The data are expressed as percentage of colony growth in control cultures with no added conditioned medium. The results shown represent the mean ± SEM of three experiments.

FCM$_{\mathrm{M}}$ was equal to that of our most potent source of CSA, HPCM. Addition of FCM or MCM to plasma clot cultures supported the growth of only four BFU-E per 10^5 cells. Thus, there was a small amount of BPA produced by unstimulated marrow stromal cells and by monocytes. Conditioned media from MFSC cultured in MCM (FCM$_{\mathrm{M}}$) produced a fourfold increase in BFU-E growth over that observed with FCM or MCM and a 15-fold increase in BFU-E over that observed in control cultures with no added conditioned medium (Table 2). Potentiator experiments revealed no increment in CSA or BPA when FCM and MCM were incubated together for 24 hours and tested for growth factor content (four experiments, data not shown). The results of assays for inhibitors of granulopoiesis and erythropoiesis are given in Table 3. No hematopoietic inhibitors were detected in any of the conditioned media tested. These results demonstrate that marrow fibroblastoid stromal cells, when stimulated by soluble products of autologous monocytes, become potent sources of CSA and BPA.

Dose–response experiments were done in which 1%, 5%, and 10% MCM, FCM, and FCM$_{\mathrm{M}}$ were added to cultured target cells in assays for BPA (Fig 1) and CSA (Fig 2). A clear dose–response relationship was seen, particularly for CSA (Fig 2). FCM$_{\mathrm{M}}$ at concentrations of 5% and 10% was significantly more stimulatory to BFU-E and CFU-GM growth than MCM or FCM. The growth factor activity of 1% FCM$_{\mathrm{M}}$ did not differ from the growth factor activity of MCM.

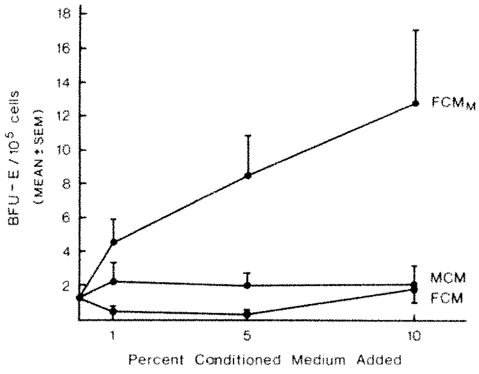

Fig 1. Dose–response curve of the BPA content of MCM, FCM, and FCM$_{\mathrm{M}}$. Conditioned media were added at a final concentration of 1%, 5%, and 10% to 1 × 10^6 nonadherent peripheral blood mononuclear cells cultured in plasma clots. The data from four experiments (MFSCs obtained from four normal donors) are shown and expressed as mean colony growth ± SEM. FCM$_{\mathrm{M}}$ 5% and 10% contained more BPA than MCM or FCM ($P < .05$).

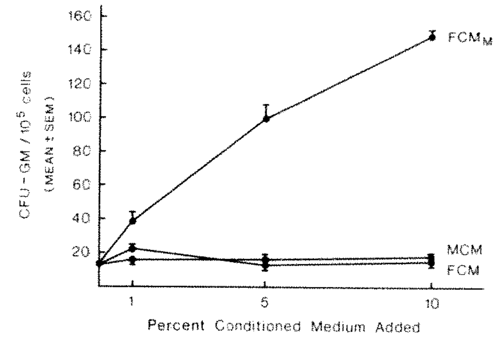

Fig. 2. Dose–response curve of the CSA content of MCM, FCM, and FCM$_{\mathrm{M}}$. Conditioned media were added at a final concentration of 1%, 5%, and 10% to 1 × 10^6 nonadherent T-depleted marrow mononuclear cells cultured in methylcellulose over an agar underlayer. Two experiments were done. The results of one experiment are given, and the data are expressed as mean number of colonies ± SEM from triplicate plates. FCM$_{\mathrm{M}}$ 5% and 10% contained more CSA than MCM or FCM ($P < .05$).

To further investigate the response of MFSCs to the monokines, varying amounts of autologous MCM (5%, 10%, 20%, and 50%) were added to MFSC cultures established from two normal donors. As little as 5% MCM stimulated a sevenfold increase in CSA production (Fig 3) and a threefold increase in BPA production (data not shown) by the MFSCs. Maximal recruitment of growth factor production was observed when the MFSCs were cultured with 10% to 20% MCM (Fig 3).

DISCUSSION

The results of our studies document that the constitutive production of CSA and BPA by human MFSCs is minimal,

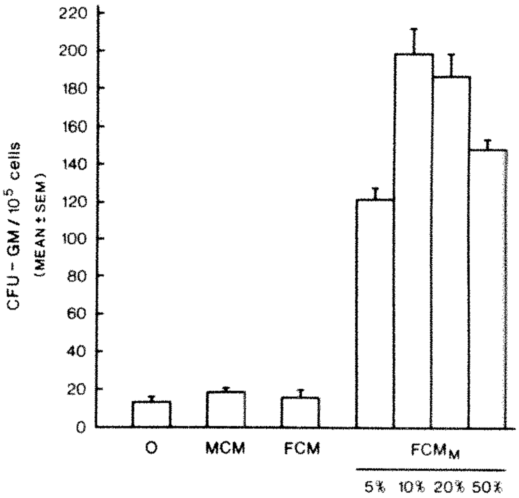

Fig 3. Response of MFSCs to varying concentrations of MCM. MFSCs were cultured in media containing 5%, 10%, 20%, and 50% autologous MCM, and the MCM, FCM, and FCM$_{\mathrm{M}}$ were assayed for CSA. Two experiments were done. The results of one experiment are expressed as mean number of colonies ± SEM of triplicate plates. All concentrations of MCM tested stimulated a significant increase in CSA production by MFSCs ($P < .05$).

but that such cells become potent producers of both growth factors when exposed to activities contained in MCM. We have referred to these activities as monocyte-derived recruiting activities. An adherent stromal cell strain established from human fetal liver was recently demonstrated to constitutively produce BPA.[22] Although certain murine marrow stromal cell lines constitutively produce CSA,[23-26] a number of groups have failed to detect CSA in conditioned media of either murine[27] or human[17] marrow stromal cell cultures. Those studies that have reported CSA production by such cells[20,28] have performed CSA assays in a system that, because macrophages were not removed, was incapable of distinguishing between constitutive production of CSA and "macrophage-recruited"[2] production of CSA by fibroblasts.

Studies of human and murine long-term marrow culture have clearly shown that an adherent layer of "stromal" cells is required for the maintenance and proliferation of progenitor cells.[29-34] While it is known that the adherent layer consists of heterogeneous cell types, including endothelial cells, fibroblasts, fat cells, and macrophages,[8,9] the mechanisms by which these cells support hematopoiesis is obscure.

Erythroid and granulocytic maturation occurs in proximity to macrophages in long-term cultures[9,35] and in association with macrophages[36] and adventitial reticular cells[16] in intact marrow, but survival of progenitor cells cannot be supported by either macrophages or fibroblasts alone. Indeed, as we have shown in the past, neither monocytes nor stromal cells, when cultured by themselves, produce substantial amounts of CSA or BPA, at least in three-day cultures.[2,3,5-7] However, as we have shown, monocytes are potent recruiters of multilineage hematopoietic growth factor production. Thus, although macrophages may produce small quantitites of factors that directly stimulate hematopoietic progenitor cells, macrophages probably play their most dominant role in regulating hematopoiesis by recruiting other cells of the hematopoietic microenvironment to produce multilineage growth factors.

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Hexachlorocyclohexanes, Potent Stimuli of O_2^- Production and Calcium Release in Human Polymorphonuclear Leukocytes

By Douglas B. Kuhns, Sandra S. Kaplan, and R.E. Basford

α-, γ-, and δ-Hexachlorocyclohexane (HCCH), but not the β-isomer, are shown to be potent stimuli for the production of superoxide anion (O_2^-) and the release of calcium in human polymorphonuclear leukocytes (PMNs). Although O_2^- production occurs in the absence of exogenous divalent cations, calcium (0.5 mmol/L) enhances O_2^- production by 50%. In addition, γ-HCCH–induced O_2^- production is sensitive ($\text{IC}_{50}{}^{-30\text{-}40}$ μmol/L) to inhibition by the putative intracellular antagonist, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8). Furthermore, it is shown that γ-HCCH induces a marked loss of membrane-associated calcium as monitored by chlortetracycline fluorescence. This pool of calcium appears to encompass the same pool released by the formylated tripeptide, formylmethionylleucylphenylalanine (FMLP). We suggest that the HCCHs represent a new class of stimuli of O_2^- production and calcium mobilization in PMNs.

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POLYMORPHONUCLEAR NEUTROPHILS (PMNs) mediate host defense against bacterial infection through a potent arsenal of bactericidal mechanisms. Recruitment of this arsenal requires a complex series of highly regulated reactions, initiated by ligand-receptor interactions at the plasma membrane surface and culminated with killing of the ingested microorganisms. The fusion of the granules with the developing phagosome and the generation of superoxide anion, O_2^-, by way of the NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase system, are major components of the cidal mechanism.[1] Initiation of these processes is thought to depend on the mobilization of internal calcium stores.[2]

The hexachlorocyclohexanes (HCCHs), a family of chlorinated hydrocarbons structurally analogous to the various isomers of inositol, represent a new class of nonphysiologic stimuli capable of mobilizing calcium and evoking a dramatic respiratory burst in human PMNs. They have been used commercially as insecticides. γ-HCCH, analogous to *muco*-inositol and the most effective insecticide, is a rapidly acting convulsant, evoking hyperexcitability, tremors, and convulsions in mammals.[3] Subsequent studies demonstrated that γ-HCCH acts as a secretagogue of neurohormones from the corpus cardiacum of the locust, possibly by promoting the increased permeability of these cells to calcium.[4] Addition of γ-HCCH to frog muscle triggered extensive myofilament degradation and mitochondrial swelling, phenomena associated with a marked rise in the intracellular calcium concentration, $[Ca^{2+}]_i$.[5] A subsequent experiment revealed that γ-HCCH induced the spontaneous release of transmitter at the frog neuromuscular junction, indicating an apparent rise in the $[Ca^{2+}]_i$ at the presynaptic terminals.[6] This rise was thought to be derived from both extracellular influxes and release of intracellular stores. Whatever the source, it has become quite apparent that the HCCHs can markedly alter the calcium homeostasis of eukaryotic cells.

Because of their structural similarity to inositol, the HCCHs have also been postulated as competitive inhibitors of phosphatidylinositol (PI) turnover. Hokin and Brown[7] demonstrated that γ-HCCH specifically inhibited the cytidine nucleotide-dependent PI synthesis in acetylcholine-stimulated, but not in resting, guinea pig cerebral cortex slices. Similarly, Fisher and Mueller[8] demonstrated inhibition by γ-HCCH of ^{32}P incorporation into PI of phytohemagglutinin-stimulated lymphocytes. More recently, however, Parries and Hokin-Neaverson[9] have questioned the specificity of these lipophilic agents and have suggested that their inhibitory nature is due to their insertion into the hydrophobic domains of enzymes or their associated lipid microenvironment.

Meade et al[10] have demonstrated in murine macrophages that γ-HCCH (at concentrations lower than those used by Parries and Hokin-Neaverson) acts as a powerful stimulus for arachidonic acid release and as an inhibitor of ^3H-inositol incorporation into PI. In addition, these authors demonstrated a small but statistically significant increase in the levels of phosphatidic acid, suggesting that γ-HCCH was indeed modulating the turnover of the PI cycle. The amount of arachidonic acid released was more than sixfold that released by zymosan. Interestingly, the arachidonate metabolites recovered were exclusively derived from the lipoxygenase pathway. These authors did not investigate the effect of γ-HCCH on superoxide anion production or any other measure of oxygen utilization.

Because both calcium and arachidonic acid (and its lipoxygenase-derived metabolites) are thought to be involved in the activation of O_2^- production in PMNs, and because the HCCHs have been shown to induce the release of both of these second messengers, we investigated the possibility that these chlorinated derivatives of inositol might stimulate O_2^- production. In the present report, we show that the α-, γ-, and δ-isomers of HCCH are potent stimuli of the oxidative burst in human PMNs. The β-isomer has no apparent O_2^--generating capacity. The rate of O_2^- generation (as high as 14 nmol of cytochrome c reduced/min/10^6 PMNs) make these stimuli among the most potent reported in the literature. Finally, evidence is presented that γ-HCCH induces a

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The data presented are taken from the dissertation of D.B.K. offered in partial fulfillment of the requirements for the Ph.D. degree.

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marked loss of membrane-bound calcium as measured by a loss of chlortetracycline (CTC) fluorescence.

MATERIALS AND METHODS

The sources of the reagents used were as follows: γ-hexachlorocyclohexane, cytochrome c (Type VI), superoxide dismutase (bovine blood), chlortetracyline, formylmethionylleucylphenylalanine, dextran (molecular weight 249,000, industrial grade), deoxyglucose, and sodium diatrizoate from Sigma Chemical Co, St Louis; β-hexachlorocyclohexane, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride from Aldrich Chemical Co, Milwaukee; α- and δ-hexachlorocyclohexanes from Pierce Scientific Co, Rockford, Ill (a gift from Dr M. Hokin-Neaverson, University of Wisconsin, Madison), Ficoll 400 from Pharmacia Fine Chemicals, Piscataway, NJ; dimethylformamide from Fisher Scientific Co, Pittsburgh. All other chemicals were of reagent grade.

The HCCHs were dissolved in dimethylformamide as stock solutions of 0.2 mol/L. Various dilutions in dimethyl formamide (0.1 mol/L, 0.04 mol/L, 0.02 mol/L) of the stocks were made for the subsequent assays. Additions of 1 to 2.5 μL/mL were made rapidly with a 10-μL Hamilton syringe, followed by vigorous mixing to ensure homogeneous distribution. Plastic pipette tips were avoided because of reagent binding.

Cell preparation. Human PMNs were isolated from fresh citrated blood from patients with secondary polycythemia (Central Blood Bank of Pittsburgh). The condition of secondary polycythemia does not affect the responsiveness of PMNs to a variety of chemotactic or O_2^- generating agents. Briefly, a solution of dextran (6% wt/vol in saline) was added to the whole blood (1 mL of dextran solution to 5 to 10 mL of whole blood), which was allowed to settle for 45 to 60 minutes at 1 g. The leukocyte-rich supernatant was then layered onto a discontinuous Ficoll 40-sodium diatrizoate gradient (density, 1.076 to 1.078) and centrifuged at 800 g for 20 minutes at room temperature. The pellet was resuspended for five minutes in isotonic NH_4Cl (155 mmol/L) buffered with $KHCO_3$ (10 mmol/L) to lyse the erythrocytes. The cell suspension was then centrifuged at 150 g for five minutes. The cell pellet was washed twice with KRPG (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L $CaCl_2$, 0.5 mmol/L $MgSO_4$, and 4.4 mmol/L glucose). The leukocyte count on the resulting suspension was then determined with a Coulter counter (Model S-Plus IV; Hialeah, Fla). The cell suspension routinely contained >97% PMNs with few contaminating erythrocytes. Platelet contamination was usually less then one platelet per PMN. Viability as assessed by trypan blue exclusion was routinely >95%.

O_2^- production. Continuous O_2^- production was assayed by the method of Newburger et al,[11] using a Gilford single-beam spectrophotometer (DU monochromator, Beckman, Irvine, Calif) thermostatically controlled with a circulating water bath (Model FE, Haake, Saddle Brook, NJ). The temperature of the incubation mix in the cuvette (1.0 mL) was monitored with a microprobe thermometer (Markson, Phoenix). PMNs (1×10^6) were added to a reaction mixture containing 150 μmol/L cytochrome c in KRPG. Superoxide dismutase (SOD, 25 μg) was added to an identical cuvette and served as the blank. After ten minutes at 37 °C, the indicated agonist was added and the reaction monitored at 550 nm on a Model 6050 chart recorder (Gilford, Oberlin, Ohio). A millimolar extinction coefficient of 21.1 was used to determine the rate of cytochrome c reduction. Values are expressed as nmoles of cytochrome c reduced/min/10^6 PMNs.

Total O_2^- production was assayed similarly using a batch procedure. PMNs (1×10^6) in 1.0 mL of KRPG containing 150 μmol/L cytochrome c were preincubated in a polypropylene tube for ten minutes at 37 °C before agonist addition. Inhibitors, if added, were present during the preincubation. After agonist addition, the reaction was incubated further for ten minutes. The reaction was terminated by transferring the samples to an ice bath. The samples were then centrifuged for ten minutes at 800 g. The supernatant solutions were assayed at 550 nm, using a similar sample containing SOD (25 μg) as a blank. The results are expressed as nmoles of cytochrome c reduced/10 min/1 × 10^6 PMNs.

Chlortetracycline fluorescence. Aliquots of 2.5 × 10^6 PMNs in 1.0 mL of KRPG were incubated with 100 μmol/L CTC for 30 minutes at 37 °C with occasional mixing. The cell suspension was

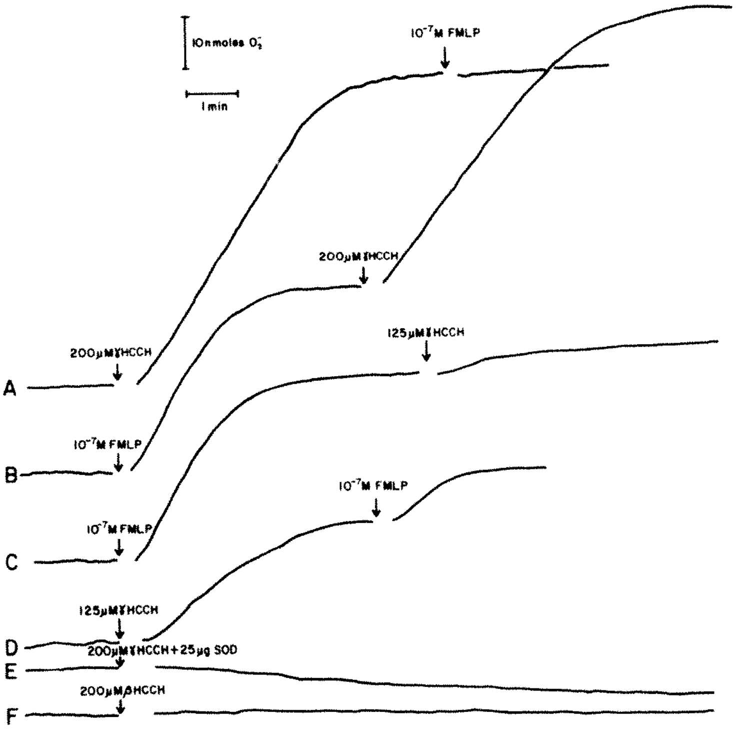

Fig 1. Superoxide generation by human PMNs stimulated by HCCHs. PMNs (1 × 10^6/mL) were preincubated for ten minutes at 37 °C before addition of the indicated stimulus. The reaction mix contained 150 μmol/L cytochrome c and was monitored at 550 nm. (Trace A) γ-HCCH (200 μmol/L) was added at the indicated arrow. A subsequent addition of FMLP (10^{-7} mol/L) is indicated by the second arrow. Interruptions in the trace represent mixing of the added stimulus. (Trace B) FMLP (10^{-7} mol/L), followed by an addition of γ-HCCH (200 μmol/L). (Trace C) FMLP (10^{-7} mol/L), followed by an intermediate dose of γ-HCCH (125 μmol/L). (Trace D) γ-HCCH (125 μmol/L), followed by an addition of FMLP (10^{-7} mol/L). (Trace E) γ-HCCH (200 μmol/L) in the presence of 25 μg of bovine superoxide dismutase. (Trace F) β-HCCH (200 μmol/L).

then centrifuged at 150 g and the cell pellet resuspended in an equal volume of KRPG. The CTC fluorescence was monitored with a spectrofluorometer (Model 430, Turner-Sequoia, Mountain View, Calif) equipped with a magnetic stirrer and automatic scanner and thermostatically regulated with a circulating water bath (Haake Model FK2). Fluorescence changes were monitored using an excitation wavelength of 390 nm (slit width 15 nm) and an emission wavelength of 515 nm (slit width 60 nm). Scans (100 nm/min) of the excitation peak (450 to 300 nm) and emission peak (600 to 450 nm) were checked before and after agonist addition to validate the results.

RESULTS

γ-Hexachlorocyclohexane proved to be a potent stimulus of O_2^- generation by human PMNs as measured by SOD-inhibitable cytochrome c reduction. Figure 1, trace A, shows that the addition of 200 μmol/L γ-HCCH resulted in a rapid release of O_2^- with a lag time of approximately 30 seconds; a further addition of 10^{-7} mol/L FMLP or of 200 μmol/L γ-HCCH (data not shown) did not reinitiate O_2^- production. The converse experiment, addition of 10^{-7} mol/L FMLP, followed by a subsequent addition of 200 μmol/L γ-HCCH, resulted in no desensitization of the γ-HCCH–induced response (Fig 1, trace B). A suboptimal concentration of γ-HCCH (125 μmol/L) induced much less O_2^- production but partially desensitized the PMNs to a subsequent addition of 10^{-7} mol/L FMLP (Fig 1, trace D). By reversing the order of additions, 10^{-7} mol/L FMLP apparently desensitized the PMNs to a subsequent suboptimal addition of γ-HCCH (Fig 1, trace C). Low concentrations of phorbol myristate acetate (3 to 50 ng/mL) and γ-HCCH (100 to 125 μmol/L) act synergistically to activate O_2^- production (data not shown). Superoxide dismutase (25 μg) completely blocked cytochrome c reduction; γ-HCCH also caused significant aggregation in the unstirred cuvette as shown by the slow decrease in absorbance at 550 nm due to changes in light scattering (Fig 1, trace E). Addition of 200 μmol/L β-HCCH caused no release of O_2^-; also no aggregation of the PMNs was observed (Fig 1, trace F). A subsequent addition of 200 μmol/L γ-HCCH resulted in a similar response as observed with γ-HCCH alone (data not shown). A dose–response curve of γ-HCCH *v* the initial rate of O_2^- production is shown in Fig 2. As high as 100 to 120 μmol/L γ-HCCH stimulated little O_2^- production; however, at 150 μmol/L, the rate of O_2^- production is maximal. As seen from Fig 2, maximal rates as high as 14 nmol/10^6/min were obtained.

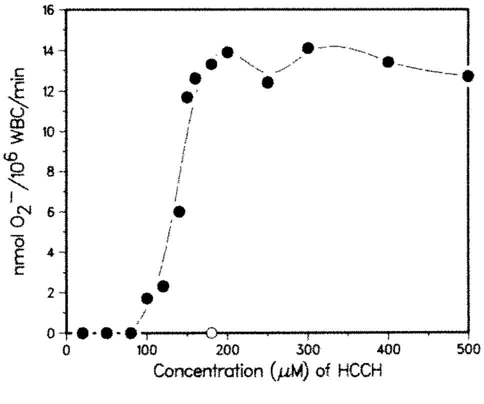

Fig 2. Effect of β- (○) and γ-HCCH (●) on the initial rate of superoxide production. Initial rates were determined as described in Newburger et al.[4]

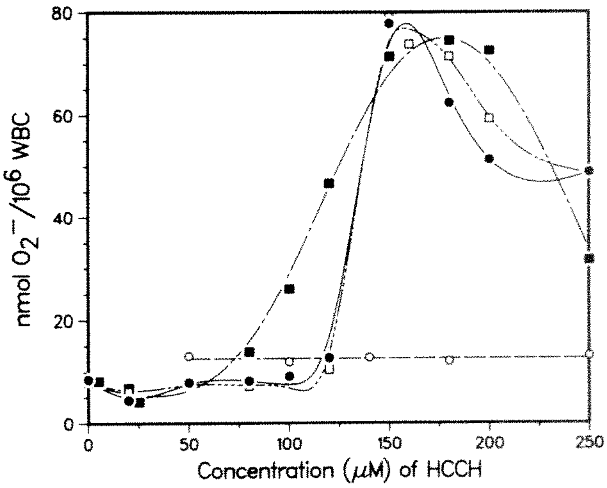

Fig 3. Effect of α-, β-, γ-, and δ-HCCH on total superoxide production. PMNs (1 × 10^6/mL) were preincubated ten minutes at 37 °C before addition of stimulus. After the addition of the appropriate HCCH, the sample was further incubated for ten minutes and then placed on ice and centrifuged. The supernatants were assayed for SOD-inhibitable cytochrome c reduction. The data are expressed as nmoles O_2^-/10^6 PMNs/10 min. α-HCCH, ●; β-HCCH, ○; γ-HCCH, □; and δ-HCCH, ■.

The total O_2^- generating capacity of the various HCCH isomers is presented in Fig 3. α-, γ-, and δ-HCCH stimulate the release of 70 to 80 nmol of O_2^- in a ten-minute incubation at 37 °C. Dose-related values of total O_2^- production can be obtained with δ-HCCH, while α- and γ-HCCH appear to yield all or no responses. At concentrations >250 μmol/L, the solubility of the isomers was exceeded and variable results were obtained. β-HCCH was found not to be an effective stimulus of O_2^- production at any concentration

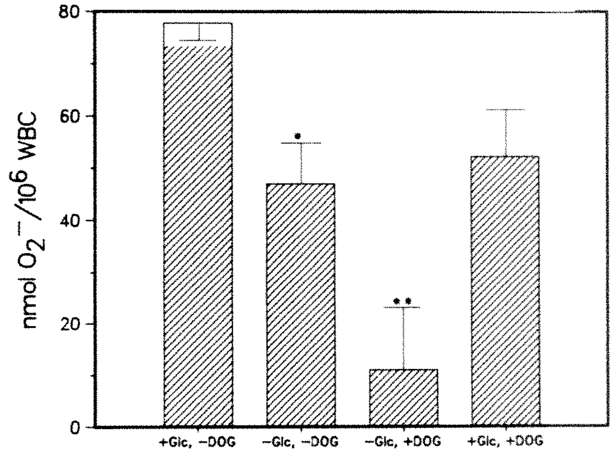

Fig 4. Requirement of glucose for γ-HCCH stimulated superoxide production. PMNs were preincubated for ten minutes at 37 °C in the presence and absence of glucose (4.4 mmol/L) ± 2-deoxyglucose (5 mmol/L). γ-HCCH (200 μmol/L) was then added and the results determined as described in Fig 2. The mean and standard deviation of three experiments is presented. *, $P < .05$; **, $P < .02$.

tested. Addition of cytochalasin B (5 μg/mL) had no effect on the O_2^- generation of γ-HCCH. γ-HCCH in the concentration range of 50 to 250 μmol/L caused no release of lactic dehydrogenase after incubation for ten minutes and did not alter the ability of PMNs to exclude trypan blue (data not shown).

Oxygen consumption, H_2O_2 production, and hexose monophosphate shunt activity all showed similar response curves with the γ-HCCH. Addition of glucose (4.4 mmol/L) was required for maximum O_2^- production (Fig 4), while 2-deoxyglucose (5 mmol/L) inhibited the maximum response by almost 90%.

By far the most intriguing aspect of γ-HCCH activation is the divalent cation requirement. γ-HCCH induces the generation of O_2^- in the absence of external cations, as shown in Fig 5. However, addition of 0.5 mmol/L calcium enhanced O_2^- production by 50%. Magnesium ion does not appear to have any effect on O_2^- production. γ-HCCH activation of O_2^- production is sensitive to the calmodulin antagonist trifluoperazine; as little as 5 μmol/L caused complete inhibition of O_2^- production (data not shown).

γ-HCCH–induced O_2^- production was also sensitive to the putative intracellular calcium antagonist TMB-8. As seen in Fig 6, the IC_{50} of TMB-8 is 30 to 40 μmol/L, much lower than the level required for zymosan-activated serum ($IC_{50} > 100$ μmol/L).[12] As with other stimuli, there appears to be an intracellular "trigger pool" whose release is a prerequisite for O_2^- generation. Studies monitoring the intracellular calcium pool using CTC fluorescence are presented in Fig 7. Addition of γ-HCCH (200 μmol/L), trace B, showed extensive loss of CTC fluorescence, which reached a plateau after six minutes and did not return to basal levels. A subsequent addition of FMLP (10^{-7} mol/L) showed no further loss of fluorescence. The insert shows the emission scan of CTC before γ-HCCH addition, at seven minutes after γ-HCCH, and after FMLP addition; complete loss of the fluorescence is demonstrated. In Fig 7, trace A, FMLP (10^{-7} mol/L) was added initially and a loss of fluorescence, although much less extensive, was observed. A subsequent addition of γ-HCCH (200 μmol/L) again caused extensive loss of CTC fluorescence. The insert reflects the emission curves of CTC before and after the additions made. These data suggest that a common calcium pool is being released by both FMLP and γ-HCCH. More important, γ-HCCH appeared to cause a marked loss of this intracellular calcium pool. Finally, β-HCCH (200 μmol/L) was shown to cause no change in CTC fluorescence (Fig 7, trace C).

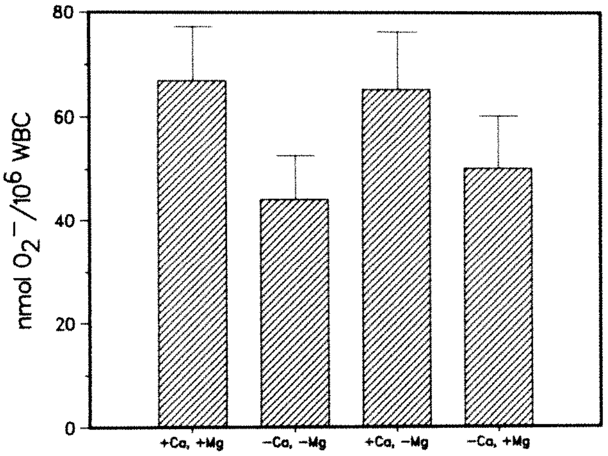

Fig 5. Divalent cation requirement for γ-HCCH–stimulated superoxide production. PMNs washed free of divalent cations were then incubated in the presence or absence of added divalent cations (0.5 mmol/L) for ten minutes before addition of γ-HCCH. Results were obtained as described in Fig 2. The mean and standard deviations of four experiments is presented.

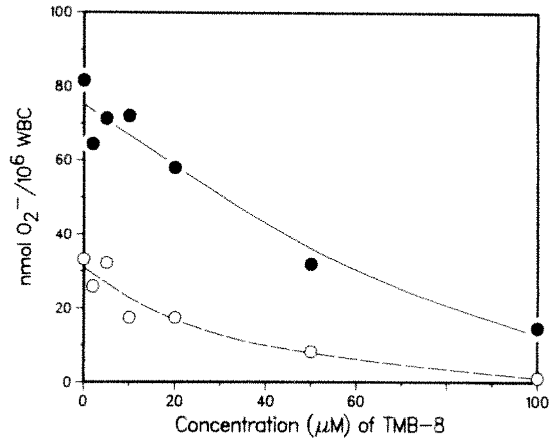

Fig 6. Effect of TMB-8 on γ-HCCH–stimulated superoxide production. PMNs ± calcium (0.5 mmol/L) were preincubated for ten minutes in the indicated concentrations of TMB-8 before addition of γ-HCCH (200 μmol/L). Results were obtained as described in Fig 2. Ca^{2+}, ●; − Ca^{2+}, ○.

The addition of γ-HCCH to quin2-AM–loaded cells[13] caused an increase in quin2 fluorescence, which represented an increase in $[Ca^{2+}]_i$ from 95 nmol/L to 180 nmol/L. Studies in the absence of extracellular Ca^{2+} also revealed an apparent doubling of $[Ca^{2+}]_i$ (data not shown).

DISCUSSION

The hexachlorocyclohexanes are potent activators of the respiratory burst in human PMNs. Differences in the effectiveness of the various isomers in generating O_2^- may not necessarily reflect structural specificity, but may be an indicator of the solubility of each compound in the membrane.[14] Interestingly, the relative toxicity of the HCCHs ($\gamma > \alpha > \delta > \beta$) is unrelated to the saturation capacity ($\delta > \gamma > \alpha > \beta$). The dose responses of O_2^- production, H_2O_2 production, and the activity of the hexose monophosphate shunt to γ-HCCH are similar. γ-HCCH–induced O_2^- production does not require exogenous glucose, but maximum O_2^- production is obtained when an exogenous source is available.

As with other activators of PMNs, calcium plays a prominent role in γ-HCCH–induced O_2^- production. An exogenous source of calcium appears to enhance the O_2^- response but is not required. Inhibition by the intracellular calcium antagonist TMB-8, however, suggests that intracellular calcium plays a significant role in this activity of the hexachlorocyclohexanes.

Our findings coincide with and confirm those of Holian et

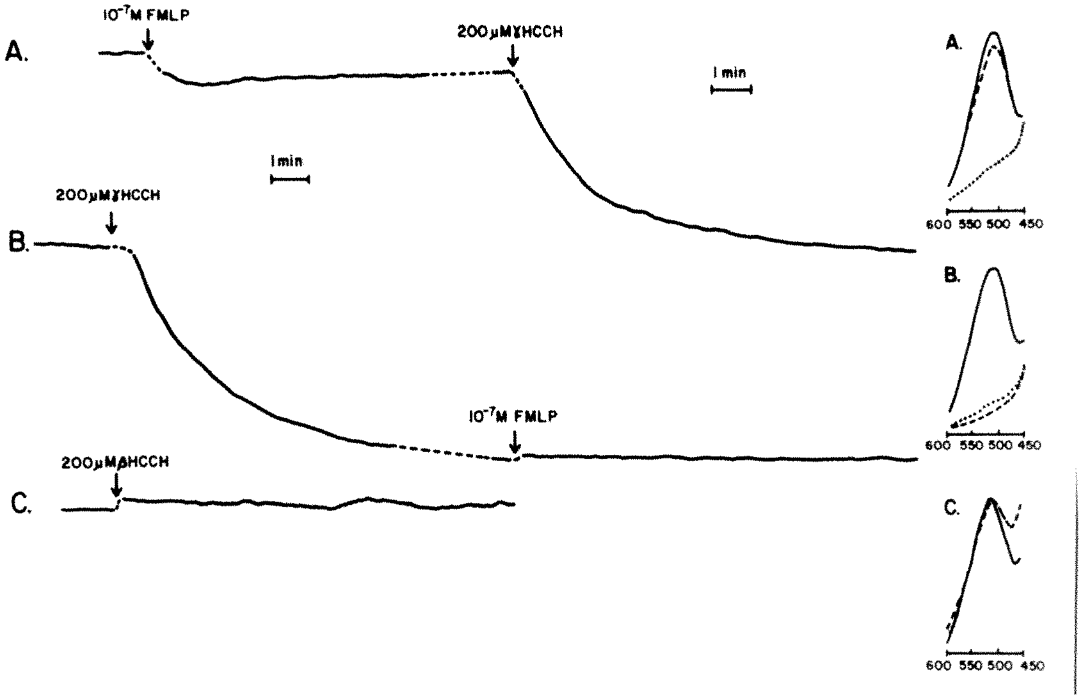

Fig 7. Effect of γ-HCCH on CTC-loaded PMNs. PMNs (2.5 × 10^6/mL) were loaded for 30 minutes at 37 °C in the presence of 100 μmol/L CTC. The PMNs were then washed once and resuspended in KRPG. The cells were then incubated an additional ten minutes at 37 °C before addition of the indicated stimulus. Dashed lines represent interruptions in the actual tracings when the additions were made or the emission peaks scanned. Fluorescence was monitored at an excitation wavelength of 380 nm (slit, 15 nm) and an emission wavelength of 515 nm (slit, 60 nm). The emission peak was scanned between 600 and 450 nm in all cases to corroborate the findings. (Trace A) FMLP (10^{-7} mol/L) was added at the indicated arrow. γ-HCCH (200 μmol/L) was subsequently added. (Trace B) γ-HCCH (200 μmol/L) addition, followed by an addition of FMLP (10^{-7} mol/L). (Trace C) β-HCCH (200 μmol/L) addition.

al,[15] who have demonstrated γ-HCCH–induced O_2^- production and calcium mobilization (^{45}Ca efflux and cytosolic [Ca^{2+}] elevation) in guinea pig alveolar macrophages. These investigators demonstrated that a minimum dose of γ-HCCH (125 μmol/L) did not desensitize the cells to a subsequent activation by FMLP (10^{-7} mol/L). Our findings support their data, but we report here that a maximum dose of γ-HCCH (200 μmol/L) results in a complete loss of subsequent FMLP-induced O_2^- production (Fig 1). A plausible explanation for these findings may be drawn from the data in Fig 7, which show that changes in intracellular calcium stores as monitored by CTC fluorescence parallel O_2^- production. The addition of FMLP (10^{-7} mol/L) caused a small but significant loss of fluorescence, indicating a release of membrane calcium. A subsequent addition of γ-HCCH (200 μmol/L) caused a further, extensive loss of membrane calcium. However, by reversing the order of additions, γ-HCCH (200 μmol/L) caused extensive loss of membrane calcium, and a subsequent addition of FMLP (10^{-7} mol/L) yielded no further loss. This would suggest that the calcium pool released by FMLP is encompassed by the same pool released by γ-HCCH. These results also parallel those of O_2^- production and suggest that there is a direct correlation between the release of membrane calcium stores and the ability to generate O_2^-.

Although the results of Parries and Hokin-Neaverson[9] cast doubt on the specificity of these agents as inhibitors of PI synthesis, we must point out that our results are obtained at concentrations ten to 20-fold less than those used in their report. The more important effect of the HCCHs may be the alterations in calcium homeostasis. Lapetina et al[16] have suggested that calcium itself may regulate the PI cycle, so the effects we demonstrated may reflect a rise in calcium and a secondary inhibition of PI cycling.

Four plausible mechanisms are suggested to explain the release of membrane calcium and production of O_2^- by the HCCHs in PMNs. (1) HCCHs, because of their highly hydrophobic nature, act as membrane perturbants, altering membrane fluidity. However, this nonspecific membrane effect is not likely, since the β-isomer had no effect on either O_2^- production or fluorescence by CTC. In addition, Jones and Lee[17] have demonstrated that γ-HCCH does interact with artificial membranes but has no detectable effect on their fluidity or permeability. (2) The HCCHs may evoke the release of endogenous mediators of calcium mobilization, specifically inositol-1,4,5-triphosphate (IP_3). Holian et al[15] have documented a drop in the polyphosphoinositides, suggesting that IP_3 may be released. IP_3 has been shown to release microsomal calcium stores in a number of different tissues, including PMNs.[18-24] (3) The HCCHs themselves are structurally related to IP_3 and may mimic the action of IP_3 in vivo. A recent report suggests that IP_3 may in fact be a second messenger for a specific IP_3-dependent kinase.[25] The HCCHs may substitute as second messengers within the PMN to induce a calcium release and a subsequent respiratory burst. (4) The HCCHs may inhibit a (Ca^{2+} + Mg^{2+})

adenosine triphosphatase (ATPase) responsible for maintaining intracellular calcium homeostasis. Jones et al[26] have shown inhibition of the (Ca^{2+} + Mg^{2+}) ATPase of sarcoplasmic reticulum by γ-HCCH. The rapidity of the calcium release induced by γ-HCCH suggests that if inhibition of an ATPase is indeed the case, the calcium homeostasis within the cell must reflect a high rate of flux to maintain homeostasis. Experiments are underway to evaluate these various hypotheses.

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High Resolution of Heterogeneity Among Human Neutrophil Granules: Physical, Biochemical, and Ultrastructural Properties of Isolated Fractions

By William G. Rice, Joseph M. Kinkade, Jr, and Richard T. Parmley

Previous studies on the fractionation of human neutrophil granules have identified two major populations: myeloperoxidase (MPO)-containing azurophil, or primary, granules and MPO-deficient specific, or secondary, granules. Peripheral blood neutrophils from individual donors were lysed in sucrose-free media by either hypotonic shock or nitrogen cavitation. Using a novel two-gradient Percoll density centrifugation system, the granule-rich postnuclear supernatant was rapidly (ten minutes) and reproducibly resolved into 13 granule fractions (L1 through L8 and H1 through H5). Granule flotation and recentrifugation experiments on both continuous, self-generated and multiple-step gradients using individual and mixed isolated fractions demonstrated that the banding patterns were isopycnic and nonartifactual. Isolated granules were intact based on the findings that biochemical latency of several granule enzymes was >95%, and thin-sectioned electron micrographs demonstrated intact granule profiles. Biochemical analyses of the granule marker proteins MPO, β-glucuronidase, lysozyme, and lactoferrin indicated that a number of the fractions were related to the major azurophil and specific granule populations. Lactoferrin was found in ten of 13 fractions (L1 through L8, H1 to H2), whereas MPO was found in every fraction. Consistent with these biochemical data, all fractions exhibited varying degrees of heterogeneity based on ultrastructural morphology and cytochemistry, including diaminobenzidine (DAB) reactivity for peroxidase and periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining for complex glycoconjugates. A variable but significant percentage (23% to 70%) of the granules in fractions L1 through L8 and H1 and H2 showed DAB reactivity, while about 90% of the granules in fractions H3 through H5 were peroxidase positive. These results demonstrated that DAB-reactive granules spanned the entire range of granule size and density. Ultrastructural PA-TCH-SP staining of isolated granule fractions revealed patterns similar to those of granules in intact neutrophils at different stages of development. Granules from human acute promyelocytic leukemia cells (HL-60) exhibited a surprisingly low density compared with typical azurophil granules from normal, mature neutrophils. The data suggest that both functional and maturational differences contribute to granule heterogeneity, and provide a new practical and conceptual framework for further defining the phenomenon of neutrophil granule heterogeneity.

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NEUTROPHILS FUNCTION AS mediators of phagocytosis and inflammation, and therefore play a major role in host defense.[1] Central to these functional roles are the neutrophil's cytoplasmic granules. These granules contain a broad spectrum of hydrolytic enzymes and microbicidal factors that function at both intracellular and extracellular sites.[2] Two major types of granules, azurophil (primary) and specific (secondary), have been characterized in human neutrophils based on ultrastructural morphology and cytochemistry[3] and separation by sucrose and Percoll gradient centrifugation procedures.[4-9] Azurophil granules have been characterized by their content of myeloperoxidase (MPO) and lysosomal enzymes, such as β-glucuronidase (βGU). The most common markers for specific granules have been the non-heme iron protein lactoferrin (LF) and a vitamin B_{12}-binding protein.[5,7] Lysozyme (LZ) occurs in both azurophil and specific granules and has been used to identify one or the other granule population under appropriate conditions.

A growing body of both biochemical and ultrastructural evidence indicates considerably greater granule heterogeneity than is generally accepted.[10] For example, a number of studies have shown that acid hydrolases do not band as a single granule component during sucrose gradient centrifugation,[5,6] and studies of the kinetics of degranulation indicate that MPO and βGU are in different granules.[11,12] More recently, we have demonstrated distinct forms of MPO[13] that are differentially distributed in different azurophil granule subpopulations[14] and that are under separate secretory control.[15] Ultrastructural studies have defined at least three morphologically distinct granule populations, the synthesis of which is associated with three successive stages of cell maturation.[16] Different stages of maturation have also been identified within the azurophil granule population,[17] and these stages have been associated with changes in both granule architecture and staining properties due to the differential insertion and/or masking of granule components.[18-20] Taken together, these data strongly suggest that both functional and maturational differences may contribute to heterogeneity within the major granule populations.

A more complete understanding and resolution of the nature of the heterogeneity of neutrophil granule subpopulations has been hampered by an inability to physically resolve the larger number of granule subtypes suggested by these biochemical and ultrastructural studies. In this report, we describe the rapid and reproducible isolation of 13 granule fractions from normal, mature human peripheral blood neutrophils using isopycnic centrifugation in Percoll density gradients. Studies of biochemical and ultrastructural properties of these fractions indicate that the size and density distribution of peroxidase-positive and -negative granules are not entirely consistent with a simple two-granule model. In addition, our results provide evidence that both functional and maturational differences contribute to the observed

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heterogeneity of neutrophil granules. Portions of this work have been published previously in preliminary communications.[21-23]

MATERIALS AND METHODS

All investigations using human material were performed with the approval of the Human Investigations Committee, Emory University, in accordance with an assurance filed with and approved by the Department of Health and Human Services.

Preparation of Granules

Human neutrophils were obtained by continuous-flow leukapheresis from normal, healthy male and female adults giving informed consent. Most experiments used cells from a single donor to eliminate possible heterogeneity resulting from genetic diversity. Residual erythrocytes were removed by two cycles of hyptonic lysis, with a resulting purity of >95% neutrophils.[24] Eosinophils were absent in leukapheresed blood samples due to pretreatment with steroids (dexamethasone). In some cases, peripheral blood was obtained by routine phlebotomy, and neutrophils were recovered in >97% purity after HESPAN sedimentation, centrifugation through LSM, and hypotonic lysis as previously described.[24] Human leukemic HL-60 cells were cultured as described previously,[25] except that they were supplemented with 10% defined bovine calf serum (HyClone). Cell staining, identification, and counting were carried out as previously reported.[24] All subsequent preparation and fractionation procedures were at 4 °C unless stated otherwise. Purified neutrophils were resuspended at a concentration of 1×10^8/mL in ice-cold hypotonic buffer (10 mmol/L Tris-HCl, pH 7.5, 0.84 mmol/L NaCl, 9.2 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1.44 mmol/L β-mercaptoethanol, 5 units heparin per milliliter) and allowed to swell in an ice bath for about ten minutes. The cells were gently lysed using a hand-held Teflon-glass homogenizer. The procedure was continually monitored by phase contrast microscopy to ensure maximal lysis (75% to 85%) without significant nuclear damage. Isotonicity was restored by adding one-tenth volume of hypertonic buffer (230 mmol/L Tris-HCl, pH 7.5, 102 mmol/L NaCl, 1.12 mol/L KCl, 40 mmol/L $MgCl_2$, 71.8 mmol/L β-mercaptoethanol), and the lysate was centrifuged for four minutes at 80 g. The resulting postnuclear supernatant (PNS) was used as a source of cytoplasmic granules. Alternatively, neutrophils were disrupted by nitrogen cavitation exactly as described by Borregaard et al,[9] and the resulting PNS was used as the source of granules.

Fractionation of Granules

All gradient solutions contained 5 units heparin per milliliter and were made isotonic by a 1:10 dilution with ten times concentrated Dulbecco's phosphate-buffered saline (PBS) and adjusted to pH 7.4 with 6N HCl, producing a 90% Percoll solution (refractive index = 1.3518). Several of the Percoll solutions had densities greater than the commercially available stock (Pharmacia Fine Chemicals, Piscataway, NJ, and Sigma Chemical Co, St Louis). The stock was concentrated by immersing a dialysis tube filled with the solution in dry Sephadex G-150. Solutions of desired density were prepared by further dilution with PBS. Densities were determined by refractive index using a calibration curve prepared by weighing Percoll solutions of known volume.

A variety of continuous, self-generated Percoll density gradients were used for studies of granule fractionation. In these studies, the PNS was mixed with different initial concentrations of Percoll and centrifuged at different relative centrifugal forces for varying periods. In the example shown in Fig 1, 0.3 mL of PNS was mixed with 8.0 mL of 79% Percoll (7 mL 90% Percoll plus 1 mL PBS) and centrifuged in a 15-mL Corex tube for 60 minutes at 27,200 g in a JA-20 rotor (34° fixed angle, Beckman, Palo Alto, Calif).

Granule fractionations on multiple-step gradients were carried out in 50-mL polycarbonate centrifuge tubes. The two gradients reported in this study were the low-density gradient (L-gradient) and the high-density gradient (H-gradient). During centrifugation, the density profiles of the multiple-step gradients rapidly fused such that shallow, essentially linear gradients were formed over a major portion of the tubes. Details for the preparation of H- and L-gradients are given in Table 1. The lowest-density layer (top) in each gradient system was the sample layer. This corresponded to a maximum of 2.5 mL of PNS from 2.5×10^8 cells made to the indicated volume with isotonic Percoll. Dilution of the PNS in the sample layer with isotonic Percoll helped to prevent aggregation by decreasing granule concentration. Also, by more nearly equalizing the densities of the sample and its adjacent layer, a transient increase in granule concentration at the interface was avoided. Centrifugations were carried out using a microprocessor-controlled Beckman J2-21M centrifuge and a JA-20 rotor (34° angle). The instrument was programmed to run at 39,200 g for ten minutes, thus providing constant conditions from experiment to experiment. Gradients were fractionated by slowly pumping (1 mL/min) the gradient from the bottom of the centrifuge tube using a blunt-ended, 20-gauge, stainless steel spinal needle. Usually about 75 samples (0.5 mL) were collected from each gradient.

Granule Extraction and Biochemical Analyses

Granule proteins from each gradient sample were extracted by the addition of 0.15 mL of PBS containing 3% cetyltrimethylammonium bromide (CTAB), 10 mmol/L EDTA, and 100 μg/mL each of 1-L-tosylamide-2-phenylethylchloromethyl ketone and phenylmethylsulfonyl fluoride. Samples were then made to a final volume of 1.5 mL with PBS, and the granules were lysed by five cycles of freezing and thawing in an acetone-dry ice bath. Under these conditions, the Percoll precipitated and was removed by centrifugation at 27,200 g for 15 minutes. The resulting supernatant containing the granule proteins (mean recoveries ± SD varied from 92% to 103%) was assayed as described below. Alternatively, intact gran-

Table 1. Preparation of H- and L-Gradients

Gradient	Layer	Volume (mL)	Density (g/cm³)	RI	90% Percoll (mL)	Diluent (mL)
L	top	4	1.045	1.3409	1.500	2.50(PNS)
	1	4	1.050	1.3416	1.681	2.319
	2	4	1.060	1.3432	2.032	1.968
	3	4	1.065	1.3439	2.207	1.793
	4	3	1.072	1.3450	1.845	1.155
	5	4	1.080	1.3462	2.737	1.263
	6	4	1.085	1.3469	2.908	1.092
	7	4	1.093	1.3481	3.188	0.812
	8	4	1.100	1.3491	3.431	0.569
	9	2	1.110	1.3506	1.889	0.111
	bottom	3	1.117	1.3517	3.000	0.000
H	top	5	1.059	1.3430	2.500	2.50(PNS)
	1	7	1.100	1.3491	5.866	1.134
	2	7	1.110	1.3506	6.479	0.521
	3	7	1.117	1.3517	7.000	0.000
	4	7	1.128	1.3535	7.000*	0.000
	bottom	7	1.142	1.3554	7.000*	0.000

Each layer contained 5 units heparin per milliliter. Except for the top layers, the diluent was PBS. RI, refractive index.

*These layers are derived from concentrated Percoll.

ules essentially free of Percoll were required for latency experiments and electron microscopy. This was accomplished by centrifuging appropriately pooled samples for three hours at 100,000 g in a Beckman SW 50.1 rotor. The granule pellet was removed from atop the hard Percoll pellet, washed with PBS, and repelleted by centrifugation at 27,200 g for 15 minutes in a Beckman JA-20 rotor.

MPO and βGU activities were measured as described elsewhere.[15] One unit of MPO activity (electron donor = guaiacol) was defined as the amount of enzyme required to produce an increase of one absorbance unit (470 nm) in one minute under the conditions described. One unit of βGU activity was equivalent to 1 μmol phenolphthalein released per minute at 37°C, pH 4.5. There was no observed inhibition of βGU due to heparin under our conditions, nor due to Percoll, since the Percoll was precipitated from samples on the addition of CTAB. MPO was also identified and/or quantified by two other methods: (1) Ouchterlony double diffusion,[26] using a monospecific rabbit antihuman MPO (IgG fraction), as described previously[27]; and (2) measurement of the reduced (dithionite) minus oxidized difference spectrum (472 nm peak), as reported by Borregaard et al.[9] LF was measured by single radial immunodiffusion[28] using rabbit anti-LF made against purified human LF.[29] Lysozyme activity was determined using the lysoplate method of Osserman and Lawlor[30] with egg white lysozyme as a standard. Protein was measured according to Lowry et al,[31] using bovine serum albumin as a standard. Cholesterol was determined according to Allain et al.[32] Total phospholipids were extracted as described by Bligh and Dyer,[33] and phosphate was measured as described by Duck-Chong.[34] Protein samples from isolated, washed, and detergent-extracted granule fractions (as described earlier) were electrophoresed into 7.5% polyacrylamide gels in the presence of SDS,[13] and silver stained.

Latency of Granule Enzymes

Granule integrity was assessed by determining the amount of several granule enzymes in the postgranule supernatant during granule isolation and by measuring the latency of these same granule enzymes. Freshly prepared PNS was centrifuged at 27,200 g for 15 minutes at 4 °C and the postgranule supernatant and granule pellet were recovered. Myeloperoxidase, LZ, and βGU were quantitated in the postgranule supernatant, as described earlier. Granule latency of these same three enzymes, as well as that of total protein, was determined according to the following relationship:

$$\%\ \text{latency} = \frac{b - a}{b} \times 100,$$

where b and a were the amount of protein or enzyme released from granules in the presence (b) and absence (a) of 0.1% Triton X-100 (Mallingkrodt, Paris), respectively.

Electron Microscopy

Granules were separated from Percoll by high-speed centrifugation and washed with PBS as described earlier. The granule pellet was overlayed with three drops of autologous plasma and several drops of 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.35. After standing at 25 °C for ten minutes, the granule-containing plasma plug was removed and fixed for an additional 50 minutes in fresh glutaraldehyde–cacodylate buffer. The plug was washed in 0.1 mol/L cacodylate buffer, pH 7.35 containing 7% sucrose, subsequently minced with a razor blade, and rinsed in cacodylate–sucrose buffer. Samples for peroxidase staining were incubated for 30 minutes in substrate medium consisting of 3 mg of diaminobenzidine (DAB) in 10 mL of 0.05 mol/L Tris-HCl, pH 7.6, to which three drops of 3% H_2O_2 were added immediately before use.[35] Control samples were incubated in medium lacking H_2O_2. Specimens processed for routine morphology and peroxidase staining were postfixed with 1% OsO_4 in 0.1 mol/L cacodylate buffer, whereas those processed for glycoconjugate staining were not postfixed. All specimens were routinely dehydrated in graded ethanol and propylene oxide and embedded in Spurr low-viscosity medium.

Thin sections of morphological and peroxidase preparations were collected on copper grids. Morphological preparations were counterstained with methanolic uranyl acetate and lead citrate (UALC), whereas peroxidase preparations were examined without counterstaining. Sections of unosmicated specimens (5 to 7 nm) were collected on stainless steel grids for periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining as described by Thiery.[36] The sections were oxidized for 45 minutes in 1% periodate, rinsed in distilled water, and then treated with 2% thiocarbohydrazide in 20% acetic acid. Specimens were subsequently washed in acetic acid, followed by three washes in distilled water. After the last wash, the samples were exposed to 1% silver proteinate for 30 minutes in the dark and rinsed thoroughly in distilled water. Control sections were run in parallel with the periodate oxidation step omitted. All specimens were examined on a Phillips 301 electron microscope or a Zeiss EM109 electron microscope at an accelerating voltage of 60 kV.

RESULTS

Preparation and Integrity of Isolated Granules

Neutrophils were lysed by a procedure involving hypotonically induced cell swelling in sucrose-free medium, followed by gentle hand homogenization. This method has the advantage that cell disruption may be carefully and continually monitored by phase-contrast microscopy. In this way, we routinely achieved disruption of about 85% of the cells without any significant lysis of nuclei or cytoplasmic granules. This was verified biochemically by analyzing the postgranule supernatant for several granule proteins. No more than 5% of total granule MPO, βGU, and LZ were found, indicating that relatively little disruption of the granules had occurred.

Two additional criteria were used to further assess the integrity of granules isolated by the hypotonic swelling method. First, granules were pelleted from the PNS and the latency of granule-associated MPO, βGU, and LZ activities was determined in the presence of 0.1% Triton X-100. Latency for each of these three enzyme activities, as well as that of total granule protein, was found to be >95%. Second, no significant (<5%) difference spectrum for MPO could be observed in granules treated with dithionite unless they had also been treated with 0.1% Triton X-100.[9] Essentially the same latency and spectral results were obtained using either unfractionated granules pelleted from the PNS or individual granule fractions subsequently isolated by Percoll density gradient centrifugation.

Identification and Isolation of Granule Density Fractions

Continuous, self-generated gradients. Our initial approach to fractionating cytoplasmic granules in the PNS was to use a wide variety of different isotonic Percoll density gradients that were self-generated during centrifugation. After several experiments, it was evident that the spectrum of densities exhibited by the total granule population was quite broad. Values ranged from approximately 1.040 to 1.150 g/cm^3 and were considerably lower than the densities reported for neutrophil granules isolated in sucrose-containing media.[4,5] However, these lower densities were consistent with the observation that subcellular organelles isolated in isotonic Percoll media exhibit significantly lower densities than the same organelles isolated in sucrose-containing media[37] and were similar to the values reported for human neutrophil granules isolated in sucrose-free media.[9]

Figure 1 shows a representative profile obtained when the granule-rich PNS was fractionated on a gradient generated during 60 minutes of centrifugation (initial Percoll concentration was 79% of stock). The membrane fraction (M), together with two major diffuse bands (S and A), were obtained. A significant amount of light-scattering material was present in the interband regions as well. These gradients were separated into approximately 75 samples, and each was analyzed for its content of four granule protein markers: LF, LZ, βGU, and MPO. Representative biochemical profiles are shown in Fig 2. These markers identified two major regions of granules that corresponded to the two visible areas previously shown in Fig 1: (1) a lower-density region (1.000 to 1.095 g/cm^3), which contained 88%, 64%, 29%, and 27% of the total recovered LF, LZ, βGU, and MPO, respectively, and (2) a higher-density region (1.095 to 1.160 g/cm^3), which accounted for 12%, 36%, 71%, and 74%, respectively, of the total recovered LF, LZ, βGU, and MPO. Based on these marker protein analyses, it was evident that the lower- and higher-density regions corresponded, respectively, to the two major populations of granules fractionated by others using both sucrose and Percoll procedures: smaller, low-density-specific granules and larger, denser azurophil granules. It was evident from these same profiles that the specific and azurophil granule regions exhibited a marked degree of heterogeneity. The presence of multiple peaks was reproducible over many experiments, suggesting that they might represent distinct density fractions.

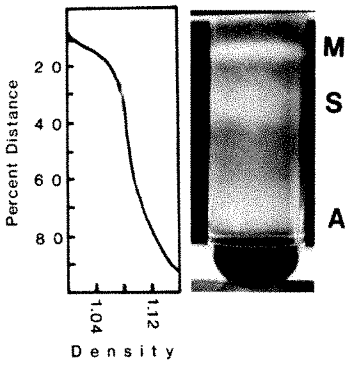

Fig 1. **Fractionation of granules using a continuous, self-generated Percoll gradient. The granule-rich PNS from 3 × 10^7 neutrophils was resolved on a continuous, self-generating Percoll gradient as described in Materials and Methods. The density profile was determined by measurement of the refractive index, and the position of each sample was expressed as the percentage of the total number of samples (75) collected. The gradient resolved three major fractions: membrane (M), specific granules (S), and azurophil granules (A). Note the presence of material in the interband regions.**

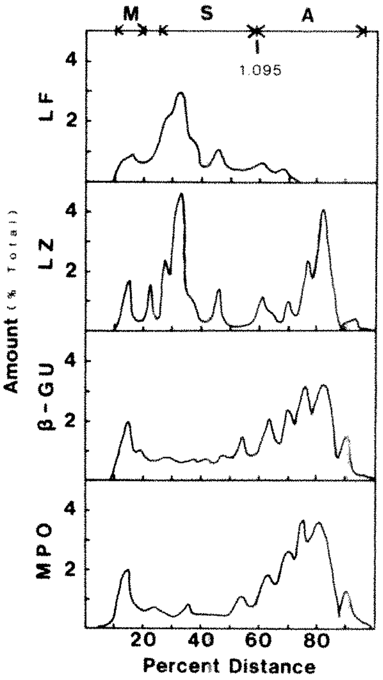

Fig 2. **Biochemical marker protein profiles of granule fractions resolved using a continuous, self-generated Percoll gradient. Lactoferrin (LF), lysozyme (LZ), β-glucuronidase (βGU), and myeloperoxidase (MPO) were quantitated in each of 75 samples obtained from a gradient identical to the one shown in Fig 1. The amount of protein or activity in each sample was expressed as a percentage of the total protein or activity recovered in the gradient. Recoveries of each marker were similar to those given in the legend to Fig 6. The position of each sample was expressed as the percentage of the total number of samples collected. M, S, and A at the top of the figure correspond to the density regions identified in Fig 1; the density 1.095 g/cm^3 shown in the top panel demarcates the two density regions corresponding to the H and L multiple-step gradients shown in Fig 3.**

Multiple-step gradients. Attempts to obtain improved resolution of these multiple peaks using a single gradient system were frustrated by constraints of commonly available centrifuge tube lengths and the dynamics of self-generated Percoll gradients.[37] In these self-generated gradients, optimization of shallowness and linearity over the low-density region was gained only at the expense of a steeper, nonlinear region in the higher density region, and vice versa. Thus, no single gradient could adequately separate all of the fractions.

In order to overcome this problem, we used a two-gradient system in which the individual gradients were designed to provide a highly resolving linear expansion of the low- and high-density regions of the granule spectrum (L- and H-gradients, respectively). Further optimization was achieved by using a series of preformed density layers, which provided several advantages over self-generated gradients. First, it allowed the construction of a shallower gradient over a greater length of the centrifuge tube. Second, because the density differences between the layers were relatively small, fusion of the layers to form an essentially linear gradient was complete in ten minutes, rather than one to two hours. Third, separation of the highest-density granule fractions in a continuous, self-generated gradient required long centrifugation periods (one to two hours) of high Percoll concentrations (>85% of stock). This resulted in formation of a large Percoll pellet at the bottom of the tube, making gradient fractionation difficult and leading to a loss of resolution between fractions. This problem was not encountered with the multiple-step H- and L-gradients. Fourth, the controlled introduction of slight discontinuities in the linear gradient resulted in a sharpening of the banding pattern, which, in turn, allowed for a more reproducible and facile fractionation of the granule bands. It is important to realize that the pattern of these minor discontinuities was specifically designed to provide increased resolution of peaks whose mean densities had already been determined on continuous, self-generated gradients such as demonstrated previously in Figs 1 and 2. A similar strategy is often used in ion-exchange chromatography, where stepwise gradients are used to minimize excessive peak tailing with eluants of fixed composition. As with such chromatographic procedures, the nonartifactuality of these gradient separations was documented by appropriate recentrifugation and mixing experiments described below.

Figure 3 shows representative density profiles and granule banding patterns obtained when a granule-rich PNS was centrifuged on high-density (H, range 1.095 to 1.150 g/cm^3) and low-density (L, range 1.040 to 1.095 g/cm^3) gradients. The uppermost band (M) near the meniscus corresponded to a membrane fraction similar to that observed by others during gradient fractionation of neutrophil PNS.[4,5] The H-gradient resolved six additional bands. Bands H1 through H5 represented five higher-density fractions, which corresponded in mean density to five peaks identified earlier by biochemical marker analyses of fractions obtained from continuous, self-generated Percoll gradients (see Fig 2, 60% to 100%). The sixth band consisted of a mixture of lower-density granule fractions (L1 through L8), which could be resolved on an L-gradient, also shown in Fig 3. On the L-gradient, the high-density bands H1 through H5 comigrated to a small region toward the bottom of the tube. Analogous to the five high-density fractions, the eight low-density fractions were also initially identified as biochemically detectable peaks on continuous, self-generated gradients. However, the more complex nature of these lower-density fractions precluded identification of all eight peaks using only a single continuous gradient such as the one shown in Figs 1 and 2.

For comparative purposes, a granule-rich PNS prepared from human promyelocytic HL-60 leukemia cells was centrifuged on an H-gradient. Surprisingly, these granules migrated as a single band near the top of the gradient. When these same granules were centrifuged on the lower-density L-gradient, they exhibited a density range of approximately 1.040 to 1.060 g/cm^3, which was much lower than typical high-density azurophil granules of normal human neutrophils (1.100 to 1.142 g/cm^3; see Fig 3).

Authenticity of Granule Density Heterogeneity

Cell lysis. A series of experiments was carried out to examine the possibility that the multiplicity of granule density fractions might be artifactual. To test whether the hypotonic lysis procedure could have contributed to the observed banding heterogeneity, we also isolated a granule-rich PNS using the nitrogen cavitation procedure for human neutrophil granules described by Borregaard et al.[9] When such cavitates were centrifuged on either the H- or L-gradient, banding patterns and mean densities were the same as those previously shown in Fig 3. Conversely, we took the granule-rich PNS isolated by our hypotonic cell lysis procedure and centrifuged it on the two-step discontinuous Percoll density gradient described by Borregaard et al.[9] As

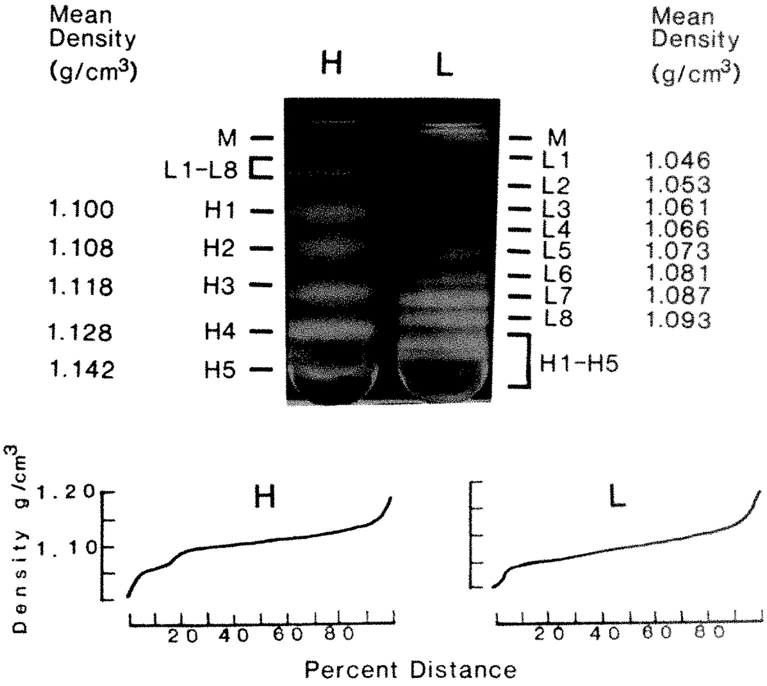

Fig 3. High resolution of neutrophil granules using high-density (H) and low-density (L) multiple-step Percoll gradients. Gradients were prepared as described in Table 1. Granules from 2 × 10^8 neutrophils were fractionated on each of the two multiple-step gradients. The H-gradient resolved five fractions (H1 through H5), and the L-gradient resolved eight fractions (L1 through L8); M designates the membrane fraction. The mean density of each fraction was determined from 25 separate experiments; ranges were never more than ±0.003 g/cm^3 from the means. Density profiles and the percentage distance were determined as described in Fig 1.

reported by these workers, we obtained three major bands, corresponding in order of increasing density to a membrane fraction, a specific granule fraction, and an azurophil granule fraction, similar to those we had observed using a continuous, self-generated Percoll density gradient (see Figs 1 and 2). These granule fractions were isolated, and the combined membrane and specific granule fractions were recentrifuged on an L-gradient; also, the azurophil granule fraction was rebanded on an H-gradient. The results of these experiments were the same as shown in Fig 3, ie, the combined membrane and specific granule fractions contained bands M and L1 through L8, while the azurophil granule fraction contained bands H1 through H5. These data demonstrated that our hypotonic lysis procedure did not produce artifactual granule fractions.

Flotation, recentrifugation, and mixing. The following experiment was designed to test whether the various granule bands had reached true isopycnic equilibrium positions or whether they were being separated on the basis of some rate zonal process. Instead of placing the granule-rich PNS on top of the preformed density layers, the unfractionated granules were placed in the lowermost, high-density Percoll layer before beginning the centrifugation. Granules were then allowed to float upward to their buoyant density positions during centrifugation. After centrifugation of both H- and L-gradients, the number of bands and their corresponding mean densities were identical to those observed using the standard procedure (Fig 3). These results verified the isopycnic nature of the fractionation procedure.

Mixing experiments were carried out to assess whether interactions between different granule populations might influence the banding profiles. A wide variety of L-fractions (single or combined fractions) were mixed with individual or variously combined H-fractions and subjected to rebanding on both H- and L-gradients. The data shown in Fig 4 are representative of this group of experiments and show the results of recentrifuging three isolated H-gradient fractions (H2, H3, H4) on an identical H-gradient (right) and a mixture of two L-gradient fractions (L5 and L6) and two H-gradient fractions (H2 and H3) on an H-gradient (left). Bands H2, H3, and H4 recentrifuged to their original mean densities (1.108, 1.118, and 1.128 g/cm^3, respectively), whereas bands L5 and L6 moved as a single band to a density position at the top of the gradient, where unfractionated L-bands were observed to run on the H-gradient (Fig 3). In these and other experiments (see below), biochemical analyses for protein, MPO, βGU, LZ, and LF confirmed the density positions of each fraction and indicated that no additional bands were present.

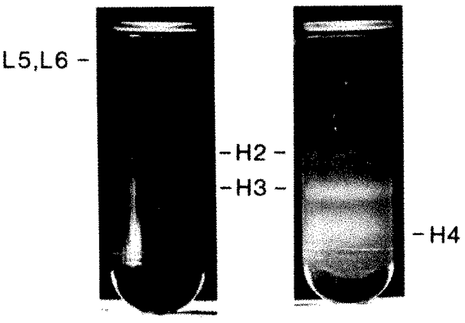

Fig 4. Recentrifugation of isolated and mixed granule fractions on a multiple-step H-gradient. Granule fractions H2, H3, and H4 were isolated individually from an H-gradient, mixed and recentrifuged on an identical H-gradient (right). Individual fractions H2 and H3 were mixed with individual fractions L5 and L6 isolated from an L-gradient and recentrifuged on an H-gradient (left). In each case, fractions H2, H3, and H4 banded at their characteristic density positions (cf. Fig 3), whereas fractions L5 and L6 recentrifuged, as predicted, in a single band.

It was important to be certain that density fractions isolated on the preformed, multiple-step gradients were not artifacts generated by the procedure itself. To investigate this possibility, various individual fractions were isolated from both H- and L-gradients and were then recentrifuged on a continuous, self-generated gradient under conditions identical to those previously described in Fig 1. Photographs and a representative biochemical marker protein profile for a gradient from this group of experiments are shown in Fig 5. Band H2, initially isolated from an H-gradient (H, left photograph) appeared as a single band at the identical mean density (1.108 g/cm^3) when recentrifuged on a continuous, self-generated gradient (S, right photograph). Biochemical analyses for βGU and MPO indicated essentially quantitative recovery of both marker proteins in a single symmetrical peak, further confirming the characteristic mean density of fraction H2 and the fact that no other granule bands were present. It should be noted that the shape of Percoll gradients (self-generated and preformed) is a function of, among other factors, the initial Percoll concentration, the time of centrifugation, and the relative centrifugal force. For this reason, as shown in Fig 5, band H2 appeared at different relative positions in these two gradients, even though these positions corresponded to the identical measured mean density.

Distribution of Biochemical Markers in Granule Fractions

H-gradient. Granules were separated using both H- and L-gradients, and the amounts of granule-associated LF, LZ, βGU, and MPO were quantified across each gradient (approximately 75 samples per gradient). Representative biochemical profiles are shown in Figs 6 and 7, and the amount of each protein or activity in a particular sample was expressed as a percentage of the total amount of that protein–activity recovered in all samples of the gradient. Total recoveries of marker proteins or activities in the H- and L-gradients, expressed as the percentage recovered (mean ± S.D.), were MPO (103 ± 5, n = 15), βGU (92 ± 7, n = 15), LZ (99 ± 6, n = 15), LF (93 ± 8, n = 9), and protein (96 ± 3, n = 4). In Fig 6, the three highest-density fractions obtained on the H-gradient (H3 through H5) contained MPO, βGU, and LZ, but no LF. This composition was characteristic of the typical high-density MPO-containing azurophil granules described by other investigators and indicated that the H-gradient provided an increased resolution of these granules. Furthermore, fractions H3 through H5 banded in a density range (mean 1.118 to 1.142 g/cm^3) that corresponded to the major, high-density azurophil granule region (A) observed using a continuous, self-generated gradient (see Figs 1 and 2; 73% to 100%). In contrast, the

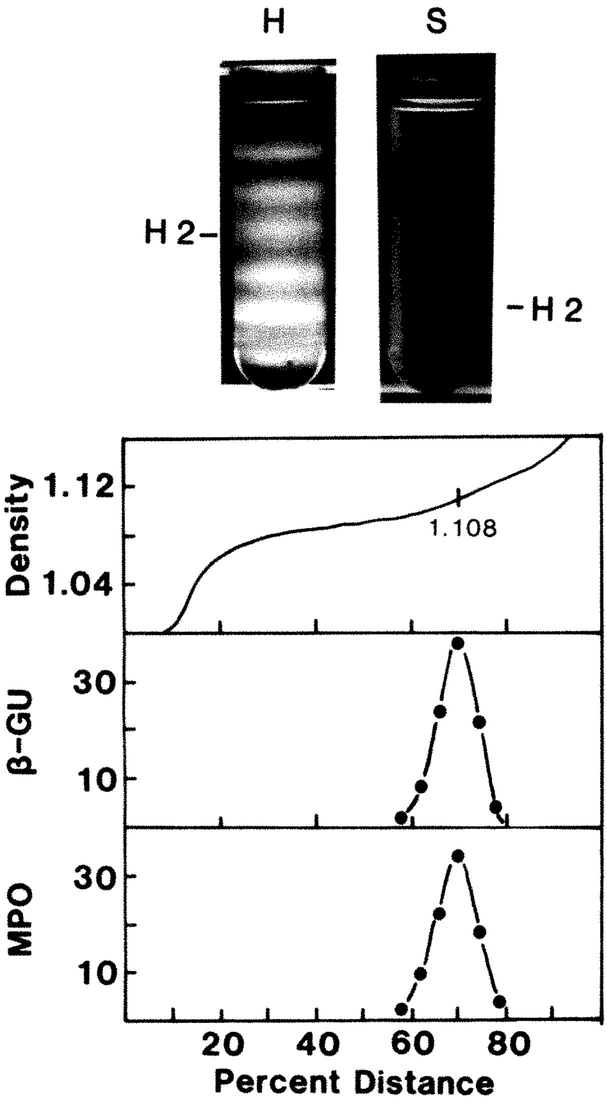

Fig 5. Recentrifugation of a granule fraction from a multiple-step gradient on a continuous, self-generated gradient. Granule fraction H2, isolated from an H-gradient (H, left), migrated as a single band when recentrifuged on a continuous, self-generated gradient (S, right) identical to the one shown in Fig 1. The upper panel shows the density profile of the gradient, and the mean density of fraction H2 (1.108 g/cm^3) is indicated by the cross-hatch. MPO and βGU activities and the percentage distance of samples were expressed as described in Fig 2. Marker protein activities were observed only in the H2 region (percentage distance, 70%), and recovery of both activities was essentially quantitative (see Fig 6).

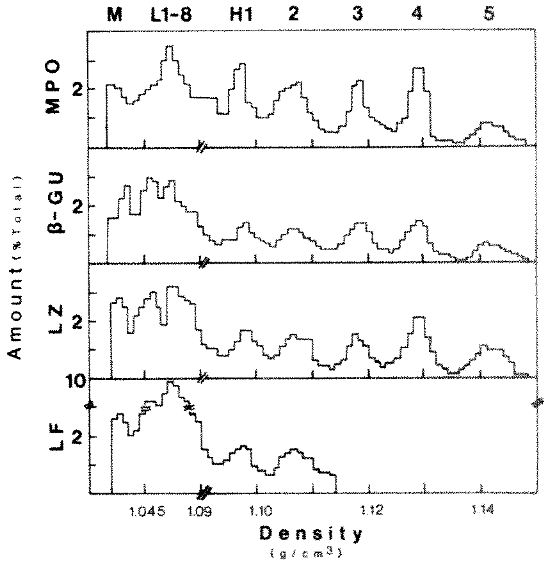

Fig 6. Biochemical marker protein profiles of high-density granule fractions separated on a typical H-gradient. Granules from 2 × 10^8 neutrophils were separated on an H-gradient to yield five high-density fractions (H1 through H5), a membrane fraction (M), and a single low-density region in which the eight low-density fractions (L1 through L8) cofractionated (cf. Fig 3). The gradient was fractionated into 75 samples and each was assayed for the marker proteins MPO, βGU, LZ, and LF. The amount of marker in each sample was expressed as a percentage of the total marker recovered in all samples, and these values were plotted as a function of the measured density of each sample. The total recovery of each marker was determined by expressing the sum of a particular marker measured in all samples of the gradient as a percentage of the known amount of that marker in the PNS that was loaded onto the gradient. Marker protein recoveries, expressed as the percentage recovered (mean ± SD), were MPO (103 ± 5, n = 15), βGU (92 ± 7, n = 15), LZ (99 ± 6, n = 15), LF (93 ± 8, n = 9), and protein (96 ± 3, n = 4).

lower-density granules (L1 through L8), which cofractionated near the top of the H-gradient, contained 85% of the total granule LF and about 30% of the total granule MPO. The relative content of these two marker proteins was essentially the same as that reported by others for specific granules. Thus, this density region contained the majority of typical LF-containing granules. Based on its biochemical profile, a third region was apparent on the H-gradient that corresponded to granule fractions H1 and H2. These two fractions contained substantial amounts of both LF and MPO, indicating a region of major overlap in which LF-containing specific and MPO-containing azurophil granules of equivalent densities cofractionated. Consistent with this view was the observation that the density range of H1 and H2 corresponded to the interband region between the specific (S) and azurophil (A) granule bands observed on continuous, self-generated gradients (see Figs 1 and 2; 60% to 73%). Together, the five high-density fractions (H1 through H5) accounted for 70% of the total granule MPO, and thus contained the majority of typical large, high-density MPO-containing granules.

L-Gradient. A representative biochemical marker profile of granules separated on an L-gradient is shown in Fig 7. The five highest-density fractions (H1 through H5) described earlier cofractionated near the bottom of the L-gradient. Fractions L3 through L8 contained 75% of the total granule-associated LF and represented subfractions of the major specific granule band (S) previously identified using a continuous, self-generated gradient (see Fig 1). These fractions also accounted for 25% of the total granule MPO. The low-density granule fractions L1 and L2 contained 5% and 10% of the total granule MPO and LF, respectively, and corresponded to granules found in the density region between the membrane (M) and specific granule band (S) of Fig 1. In general, LF was found in

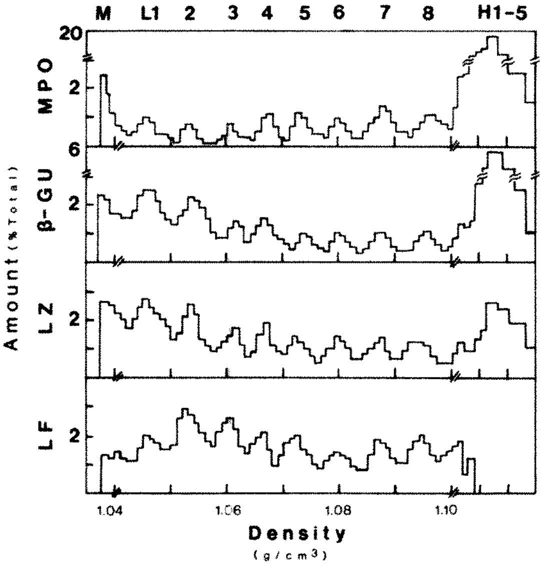

Fig 7. Biochemical marker protein profiles of low-density granule fractions separated on a typical L-gradient. Granules from 2 × 10^8 neutrophils were separated on an L-gradient to yield eight low-density fractions (L1 through L8), a membrane fraction (M), and a density region at the bottom of the gradient that contained the five high-density fractions (H1 through H5). The ordinate, abscissa, and recoveries are the same as described in Fig 6.

fractions L1 through L8 and H1 and H2, indicating that LF-containing granules spanned a major portion of the granule density spectrum (mean range, 1.046 to 1.108 g/cm^3). On the other hand, MPO (measured enzymatically, spectrally, and immunologically), βGU, and LZ were found in all granule fractions, indicating that MPO-containing granules were localized across the entire granule density range (mean, 1.046 to 1.142 g/cm^3). All four marker proteins were always observed at the top of each gradient (with the exception of recentrifuged fractions) in an area corresponding to the initial sample volume. The amounts of these proteins never accounted for more than 5% of the total recovered marker protein and probably reflected a small degree of granule damage, which occurred during the preparative procedure.

Equivalent amounts of protein from each isolated granule fraction were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). Quantitation of such staining patterns is often unreliable, particularly when dealing with a complex mixture of proteins with widely different properties. Nevertheless, the staining patterns were consistent with the quantitative biochemical data and showed MPO in all granule fractions but LF only in fractions L1 through L8 and H1 and H2. Interestingly, when the highest-density granule fraction, H5, was compared with the other four high-density fractions, it consistently appeared to be relatively deficient in MPO and elastase isoenzymes as judged by staining intensities. In addition, determination of cholesterol to total phospholipid ratios for all the granule fractions revealed a surprisingly wide range (0.62 to 1.40) and a bimodal distribution peaking in the regions of fractions L6 and H2 through H4.

It must be emphasized that varying degrees of granule heterogeneity were observed within all of the density fractions based on morphological and staining differences at the ultrastructural level (see below). For this reason, it was not possible to further extrapolate differences in the biochemical profiles between fractions to differences in the granule subpopulations themselves. However, this additional level of granule complexity was consistent with two of the biochemical observations: (1) the cofractionation of MPO-deficient–LF-containing granules with MPO-containing–LF-deficient granules in fractions L1 through L8 and H1 and H2 and (2) the finding by this laboratory and others[4] that as much as 30% and 50% of the total granule MPO and βGU, respectively, were associated with the lower-density fractions. This observation is not merely a reflection of nonspecific adsorption of MPO to LF-containing granules, but is due to the presence of smaller, low-density peroxidase-containing granules.

Electron Microscopy of Granule Fractions

Characterization of the fractions was carried out at the ultrastructural level using morphological (uranyl acetate-lead citrate [UALC]), peroxidase (DAB), and complex glycoconjugate (PA-TCH-SP) staining as distinguishing criteria. In all preparations, each of the fractions contained abundant cytoplasmic granules, most of which demonstrated intact granule membranes. There were no observed eosinophil or basophil granules and only rarely were platelet alpha granules observed. Furthermore, there was minimal contamination (<1% of organelles) with mitochondria and plasma membranes.

Morphology. There was a wide range in the mean diameter of granules from the different isolated fractions. An example of this is illustrated in Fig 8. Approximately a two-fold difference in mean diameter was observed between the granules of fraction L1 and those of fraction H2. Figure 9 shows the results of a morphometric analysis of granules in each of the 13 isolated fractions. It can be seen that there was a generally progressive increase in mean diameter from the lowest-density fraction, L1, to the highest-density fraction, H5. Determination of mean granule diameters was obtained by measurement of the largest diameter of all membrane-bound structures (n = 200), which were consecutively scored in randomly selected photographs. To prevent observer bias, no attempt was made to exclude tangentially sectioned granule profiles; consequently, the diameters may be somewhat smaller than the actual mean diameters. Nevertheless, granules in fractions L1 and L2 (mean diameter, 0.14 μm) were clearly smaller than typical specific granules (fractions L3 through L8; mean diameter, 0.21 μm) or typical azurophil granules (fractions H3 through H5; mean diameter, 0.29 μm).

Complex glycoconjugates. The PA-TCH-SP procedure has been used previously with intact neutrophils to differentiate between specific and azurophil granules, which show intense and weak staining of the matrix, respectively.[19] An example of this difference in staining intensity is shown in Fig 8. The PA-TCH-SP stain has also been used to distin-

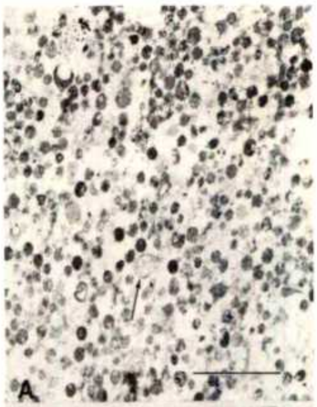

Fig 8. Ultrastructural demonstration of size and staining differences among isolated granule fractions. Granules were stained using a PA-TCH-SP procedure for vicinal glycol-containing carbohydrates. Granules from fraction L1 (panel A) had a mean diameter of 0.14 ± 0.05 μm, and granules from fraction H2 (panel B) had a mean diameter of 0.26 ± 0.07 μm. Note the intense staining of L1 granules compared with the weak staining of H2 granules. The arrow in (A) points to a larger granule that was trapped with fraction L1. Original magnification, 24,500×; bar equals 1 μm.

guish immature from mature granules of both major granule types based on differences in staining patterns seen in intact neutrophils.[19] PA-TCH-SP type I granules were presumed to be mature specific granules that exhibited intense matrix but no membrane staining, whereas type II granules corresponded to immature specific granules with intense staining of both matrix and membrane. Type III granules were presumed to be mature azurophil granules that had weak matrix and no membrane staining, and type IV granules included immature azurophil granules that showed membrane staining and weak matrix staining. Using the same staining procedure with our isolated fractions, we were able to distinguish these four types of granules, representative examples of which are shown in Fig 10: type I (fraction L2, panel A), type II (fraction L4, panel B), type III (fraction H1, panel C), and type IV (fraction H2, panel D). Figure 11 shows the distribution in each fraction of the percentage of granules with PA-TCH-SP staining typical of specific granules (types I + II, panel A) and of azurophil granules (types III + IV, panel B). Granule types I and II were predominant in the lower-density fractions (L1 through L8), whereas types III and IV predominated in the higher-density fractions (H1 through H5). This was consistent with the biochemical studies, which showed that LF-containing specific granules were enriched in the lower-density fractions and the typically large MPO-containing azurophil granules were enriched in the higher-density fractions.

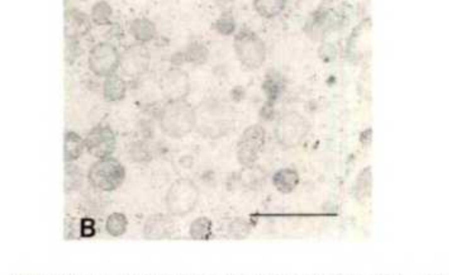

Fig 9. Relationship between mean density and mean diameter of isolated granule fractions. Mean diameters (open circles ± SD) were derived from morphometric analyses of 200 granules in each of the 13 granule fractions isolated using the H- and L-gradients. Mean densities were determined for granule fractions in 25 experiments (see Fig 3).

Peroxidase. Peroxidase staining using DAB was performed on all fractions, and the percentage of peroxidase-positive granules in each fraction (n = 200) is shown in Fig 11 (panel C). As expected from the biochemical analyses, each of the five highest-density fractions contained a high percentage of peroxidase-positive granules, but interestingly, a significant percentage of granules in the lower-density fractions also showed peroxidase reaction (range of 23% to 60%). Furthermore, there was a wide variation in the pattern and intensity of peroxidase staining among the fractions; these included homogeneously intense granules in L1, granules containing lucent crystalloids in L8 and H1, homogeneously diffuse granules in H3, and granules with a weakly rim-staining pattern in H5.[22,23]

DISCUSSION

The present study describes a novel procedure for the rapid and reproducible isolation of 13 granule density fractions from normal human neutrophils of individual donors. Our preliminary studies showed that the broad spectrum of densities characteristic of these granules precluded optimal resolution when only one gradient was used. For this reason, we developed a high-resolution two-gradient system that significantly expanded the high- and low-density regions. The new system used a series of preformed density layers of isotonic Percoll in sucrose-free media. Under the conditions of centrifugation, these multiple-step layers rapidly (10 minutes) fused to produce an essentially linear gradient. This approach resulted in the isolation of eight granule fractions (L1 through L8) using the low-density L-gradient and five granule fractions (H1 through H5) using the high-density H-gradient (Fig 3).

Both the H- and L-gradients purposely used small discontinuities in the density profiles to achieve sharpening of individual granule fractions. Nevertheless, it is important to realize that this strategy did not generate artifactual banding

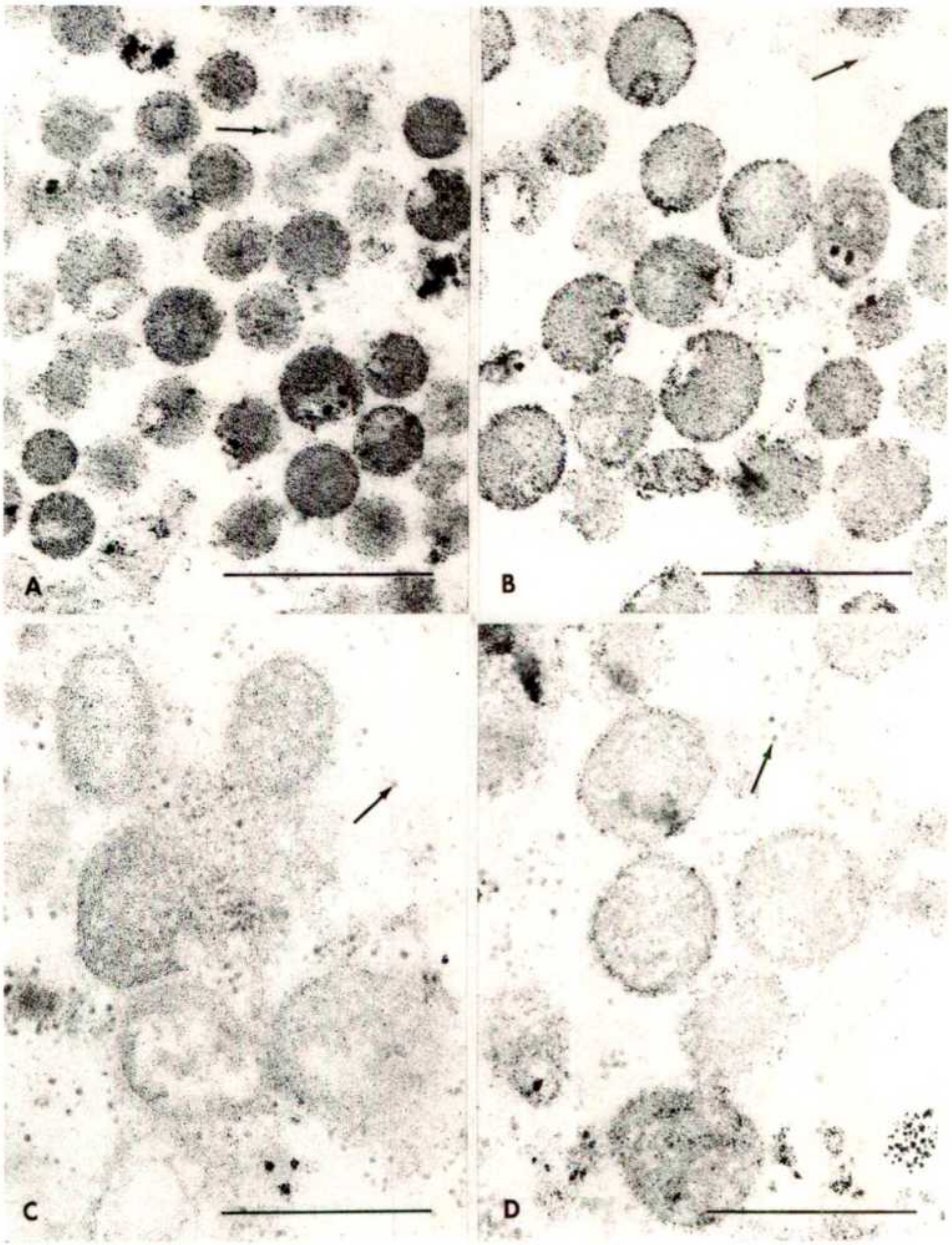

Fig 10. Identification of four types of PA-TCH-SP staining patterns in isolated granule fractions. (A) Type I granules had intense matrix and no membrane staining as in fraction L2 granules. (B) Type II granules had intense matrix and membrane staining as in fraction L4 granules. (C) Type III granules had weak matrix and no membrane staining as in fraction H1 granules. (D) Type IV granules had weak matrix along with membrane staining as in fraction H2 granules. Arrows point to residual Percoll in the granule samples. Original magnification 70,000×; bar equals 0.5 μm; current magnification 56,000×.

profiles. Indeed, granule fractions were initially identified using a variety of continuous, self-generated gradients (see Figs 1 and 2), and a large number of mixing and recentrifugation experiments (Fig 4) demonstrated that the fractions were not artifacts of the cenrtifugation procedure or due to nonspecific aggregation. These experiments included recentrifugation of granule fractions isolated using multiple-step gradients on continuous, self-generated gradients and vice versa (Fig 5). Granule fractions were also shown to result from isopycnic equilibration rather than from a rate zonal process by demonstrating that flotation of granules upward during centrifugation produced the identical density patterns as when granules were loaded on top of the gradient.

Recently, Borregaard et al[9] used Percoll as the density medium for fractionation of neutrophil granules and observed that the densities of the granules were significantly decreased compared with those isolated using hypertonic sucrose solutions. It was also shown that isolation of granules using nitrogen cavitation and isotonic Percoll in sucrose-free media yielded granules with intact membranes. This was based on the inability of exogenously added dithionite to reduce granule-associated MPO in the absence of detergent.[9]

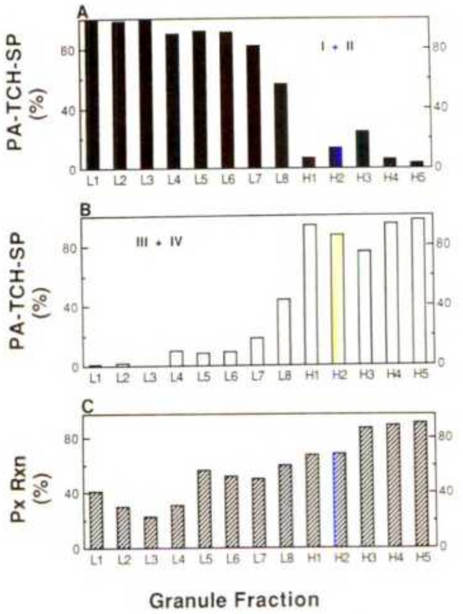

Fig 11. Quantification of ultrastructural cytochemical staining patterns typically associated with specific and azurophil granules. Isolated granule fractions from a typical experiment were PA-TCH-SP stained, and 100 granules from each fraction were scored for the percentages of the four PA-TCH-SP staining types as illustrated in Fig 10. The percentages of Types I + II (strong matrix staining, A) and Types III + IV (weak matrix staining, B) in each fraction were determined to give the total percentages of granules with staining patterns typical of specific or azurophil granules, respectively. As a positive stain for azurophil granules, isolated fractions from a typical experiment were stained with DAB for peroxidase reactivity, and 200 granules from each fraction were scored for the percentage of peroxidase positivity (C).

We have confirmed and extended these observations. We found that granules prepared from cells lysed in sucrose-free media using either nitrogen cavitation or the hypotonic lysis method gave the same two major granule fractions when separated according to Borregaard et al[9] and the same 13 granule fractions using our fractionation procedure (Fig 3). Thus, our fractions were not artifactually generated by the hypotonic cell lysis procedure. In addition, we demonstrated the intactness of isolated granule membranes using three criteria: (1) the MPO difference spectrum procedure of Borregaard et al,[9] (2) the latency (>95%) of three granule enzyme activities in the presence and absence of detergent, and (3) the observation of intact granule profiles in thin-sectioned electron micrographs. Taken together, our data strongly support the in vivo existence of substantial heterogeneity among neutrophil granules.

There is, in fact, a growing body of biochemical, cytochemical, and ultrastructural evidence that is consistent with a significant degree of granule complexity but which heretofore has not received widespread attention.[10] Ultrastructural studies have demonstrated three morphologically distinct granules that form in successive stages.[17,38-40] Peroxidase-positive azurophil granules were observed to form first (primary) during the promyelocyte stage of cytodifferentiation, while the specific granules formed later (secondary) during the myelocyte stage. It has also been reported that small lysosomal or "tertiary"granules, distinct from specific granules, are formed during the later stages of neutrophil development.[10,16,17] More recently, it has become common to designate granules with properties different from either primary/azurophil or secondary/specific granules as tertiary granules, although the temporal sequence of their formation is uncertain.

Biochemical analyses of fractions isolated primarily by sucrose density gradient centrifugation have resolved two major granule types corresponding to the peroxidase-containing azurophil granules and peroxidase-deficient specific granules.[3] On the other hand, using what are generally accepted as characteristic marker proteins, at least four classes of granules have been reported[4-6,41,42]: (1) azurophil granules (MPO, βGU), (2) specific granules (LF, vitamin B_{12}-binding protein), (3) tertiary, or small lysosomal, C-particles (acid β-glycerophosphatase, *N*-acetyl-β-glucosaminidase), and (4) phosphasomes (alkaline phosphatase). Among these granule populations, only the azurophil granule fraction has been clearly shown to contain isolated subfractions, and several studies have demonstrated the existence of two MPO-containing subpopulations using sucrose density gradient centrifugation.[4,5,14]

Other fractionation studies and kinetics of the release of granule components have suggested further granule heterogeneity and illustrate that a major problem in defining granule subfractions has been the apparent lack of specificity of some of the marker proteins. For example, MPO and βGU are both commonly used as markers of high-density azurophil granules, but these markers have also been found in all previously isolated lower-density specific granule fractions.[4,5] In addition, some fractionation studies indicate that MPO and a number of acid hydrolases exist in the same granule,[6] whereas studies of the kinetics of release of these enymes and several other fractionation studies indicate that MPO and βGU are in different granules.[4,5,11,12] Also, gelatinase has been shown to be associated with a novel, as yet morphologically unidentified secretory unit,[43] and a unique b-cytochrome,[9,44] proposed as part of the oxidase system involved in the respiratory burst,[45] was partially resolved from specific granules by rate zonal sedimentation.[46]

The resolution of a large number of granule fractions in the present study raises two fundamental questions. First, is this degree of heterogeneity completely at odds with the widely accepted two-granule model for neutrophils? And second, what is the biological significance of this heterogeneity? We believe that our results are consistent with many of the findings that support the two-granule model. Nevertheless, the isolation of a number of subfractions necessitates a reevaluation of the suitability of the two-granule paradigm for thinking about the origin and function of these cytoplasmic structures.

Using a single continuous, self-generated density gradient procedure, our granule-rich PNS was found to contain the two major granule populations (Fig 1, S and A) as reported by others.[4-7,9] Typically, the azurophil granule region contained MPO but no LF, and the specific granule region contained LF and some MPO (Fig 2). The use of a two-gradient strategy (Fig 3) allowed further resolution of both the specific and the azurophil granule regions as well as resolution of additional fractions with densities between the membrane and specific granule regions and between the specific and azurophil granule regions (Figs 1 through 3). From the MPO-containing/LF-deficient azurophil granule region, three fractions were obtained (H3 through H5; Fig 6) that corresponded to relative enrichments of several large peroxidase-positive granule subtypes observed by others in their ultrastructurally heterogeneous azurophil granule fraction.[4,17] From the specific granule region that was enriched in LF but still contained MPO, we resolved six fractions (L3 through L8; Fig 7). Fractions H1 and H2 were isolated from the density region between the specific and azurophil granule regions. The two lowest density fractions, L1 and L2, were derived from the density region between the membrane and the specific granule region. Hence, our data can be easily related to the fractionation of two major granule populations.

A number of observations in the present work do not fit a simple two-granule model, but are more consistent with the literature cited earlier that suggests greater heterogeneity of granules. The most obvious is the finding that peroxidase-positive granules were not solely restricted to the larger, high-density azurophil granule region, but rather spanned the entire ranges of size (mean diameter range, 0.14 to 0.32 μm) and density (mean density range, 1.046 to 1.142 g/cm^3) of neutrophil granules (Fig 11). Specific granules have never been isolated completely free of MPO,[4,5,9] and this has generally been attributed to contaminating azurophil granules or the adsorbed contents of damaged azurophil granules. In agreement with these previous results, our biochemical analyses of fractions L3 through L8 (which corresponded to the specific granule region) indicated that approximately 25% of the total granule MPO was associated with these fractions (Fig 6). Ultrastructural studies of these same fractions showed that, on the average, about 45% (range, 23% to 60%) of these granules demonstrated peroxidase reactivity (Fig 11). Thus, it is clear that neutrophils contain relatively large numbers of peroxidase-positive granules with sizes and densities similar to peroxidase-negative specific granules.

The distribution of peroxidase-reactive granules in the lighter-density fractions indicated that the typical correlation of this property with only high-density azurophil granules was not strictly applicable to the more complex array of isolated granule fractions. Likewise, the PA-TCH-SP staining procedure for complex glycoconjugates did not always distinguish between azurophil and specific granules. Typically, specific granules have been shown to exhibit intense matrix staining while that of azurophil granules is weak (Figs 8, 10, 11).[19] Granules of fraction H5 were consistent with this staining pattern and exhibited >90% peroxidase positivity and >90% weak matrix PA-TCH-SP staining. In contrast, virtually all granules in fraction L1 exhibited the intense matrix PA-TCH-SP staining typical of specific granules (Fig 8), but 41% of the granules also exhibited peroxidase reactivity typical of azurophil granules (Fig 11). These data define a third major type of granule (strongly reactive for both peroxidase and complex glycoconjugates) and suggest that the terms azurophil and specific are not adequate to simply describe granules that do or do not contain peroxidase.

Similar to MPO, βGU has been considered as an azurophil granule marker, but significant amounts of this enzyme also have consistently been associated with the specific granule fraction (Fig 7).[4,5] The present data do not allow us to distinguish whether βGU is colocalized with the smaller, low-density peroxidase-positive granules or whether it resides in peroxidase-negative granules, or perhaps both. However, the existence of this greater granule heterogeneity offers a possible explanation for the observations that MPO and βGU are colocalized in large, high-density azurophil granules but do not exhibit the same kinetics of secretion.[11,12]

Of particular significance in the present work was the isolation and identification of two novel peroxidase-positive granule types that appear to provide further functional correlates to granule heterogeneity. Fractions L1 and L2 were found to contain a variety of small granules, which we have termed microgranules, with mean diameters (0.14 μm) and densities (1.046 to 1.053 g/cm^3) considerably lower than neutrophil granules previously isolated. About 41% of these microgranules were peroxidase-positive (Fig 11), and based on their physical, biochemical, ultrastructural, and secretory properties, they represent a new class of DAB-reactive microgranules distinct from typical azurophil granules.[22] In addition, further examination of fraction H5 revealed that it consisted of a subpopulation (>70%) of distinctively large granules that were enriched (about 50% of total protein) in a new class of microbicidal defensin polypeptides,[47,48] but that were relatively deficient in the characteristic azurophil granule markers, MPO and elastase.[23] Because defensins are biologically active under anaerobic conditions, these newly isolated granules may play a prominent role in nonoxidative host defense mechanisms of neutrophils. Studies are in progress to determine whether these two newly identified MPO-containing granule subtypes are related to the earlier observations of Kinkade et al[13-15] regarding the differential localization and secretion of different forms of MPO.

A large proportion of peroxidase-negative granules were also observed in the region of lower size and density represented by fractions L1 through L8. Although about 85% of the total specific granule marker protein LF was found in these granule fractions (Fig 7), ultrastructural studies indicated considerable heterogeneity (Figs 8 through 11). We propose that this broad range of granules includes peroxidase-negative specific granules that typically contain LF and vitamin B$_{12}$-binding protein as well as peroxidase-negative granules that do not necessarily contain these specific granule marker proteins. These other granules may contain one or more of the recently described markers, such as gelatinase, histaminase, Mol glycoprotein, or laminin receptors, which do not appear to be associated with typical specific granules.[16,49-51] Furthermore, the elusive "tertiary" granule, or C-particle, which has been described as small relative to specific and azurophil granules and which contains acid hydrolases and glycosaminoglycans but not MPO,[6,10,16,17,38-40] may also be localized to the peroxidase-negative granules among these lower-density fractions.

The demonstration of considerable complexity among neutrophil granules raises the question as to what factors contribute to the generation of this phenomenon. A number of lines of evidence indicate that heterogeneity exists among populations of normal human peripheral blood neutrophils.[52] Presently, however, the functional implications of this observation for neutrophil biology are unclear. On the other hand, there is clear evidence that functional heterogeneity operates at the level of cytoplasmic granules.[2,13] What remains to be determined is whether granule heterogeneity reflects different granule types within subsets of neutrophils, or whether there are functionally distinct subsets of granules within all neutrophils, or perhaps a combination of the two possibilities.

A second possible contributing factor to granule heterogeneity is the existence of granules at different stages of maturation. In ultrastructural studies using a cytochemical stain for complex glycoconjugates (PA-TCH-SP), Fittschen et al[19] identified at least four maturational forms of granules in directly sampled, intact neutrophils. Using this procedure, we observed these same four PA-TCH-SP staining patterns in our isolated granule fractions (Fig 10). This observation suggests that developmental events may still be occurring in at least some of these cells and is consistent with the observation that morphologically mature bone marrow neutrophils are functionally immature compared with their peripheral blood counterparts.[53] In this regard, it is interesting to note that the azurophil granules of undifferentiated HL-60 cells, which are predominantly immature promyelocytes, were found to exhibit a significantly lower density than typical azurophil granules from normal cells (Fig 3). Whether the decreased density of the azurophil granules from HL-60 cells reflects an abnormality associated with the leukemic state or if a lower density is characteristic of normal, immature promyelocyte azurophil granules remains to be elucidated.

Third, it seems reasonable that granule heterogeneity may also reflect various aspects of intracellular membrane trafficking. During their development and life cycle, phagocytic cells, such as neutrophils, are actively involved in lysosomal, secretory, and endocytic pathways. In this regard, Fletcher and Gallin[54] have demonstrated intracellular pools of preformed receptor for the synthetic chemoattractant FMLP and for complement component C3bi. Although the ultrastructural localization of these compartments has not yet been reported, they migrate on sucrose gradients with specific granules. Additional studies are necessary to establish the possible contribution of these compartments to granule heterogeneity.

In conclusion, our results clearly support the concept of substantial heterogeneity among human neutrophil granules. Experiments are in progress to determine the banding and biochemical profiles of granule fractions from neutrophils treated with various secretagogues and should provide further insight into the functional significance of this heterogeneity. In addition, because typical azurophil and specific granules are differentially mobilized for secretion by various stimuli,[55] such studies may provide a means of further purifying MPO-containing/LF-deficient and MPO-deficient/LF-containing granules from regions of overlap (fractions L1 through L8 and H1 and H2). At the moment, it would be premature to suggest an alternative nomenclature for describing this complex array of granules. Nevertheless, there is a clear need for more carefully defining which proteins should serve as selective markers for distinguishing particular granule subpopulations. We believe that our findings provide a new approach to the task of more definitively characterizing the nature of this heterogeneity and its functional correlates in normal neutrophils and in various pathological states, such as granulocytic leukemia.[56] Also, the methodology developed for the resolution of granule heterogeneity in neutrophils may prove useful in identifying unrecognized complexity in other subcellular systems.

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Repolarization of the Membrane Potential of Blood Platelets After Complement Damage: Evidence for a Ca^{++}-Dependent Exocytotic Elimination of C5b-9 Pores

By Peter J. Sims and Therese Wiedmer

Gel-filtered blood platelets exposed to complement proteins C5b-9 have previously been shown to undergo a reversible depolarization of membrane potential (E_m) in the absence of lytic plasma membrane rupture. In this paper, we examine the mechanism by which C5b-9 damaged platelets restore their basal electrochemical state, despite increased ion conductance due to membrane insertion of these cytolytic serum proteins. Repolarization of E_m after formation of the C5b-9 membrane pore is shown to be accompanied by a Ca^{++}-dependent vesiculation of the platelet surface, which results in the release of these proteins from the plasma membrane and a restoration of the membrane's functional integrity. This exocytotic elimination of C5b-9 complexes from the plasma membrane is accompanied by a ouabain-inhibitable repolarization of E_m, which presumably reflects restoration of transmembrane cation gradients by the plasma membrane Na/K ATPase. The role of external Ca^{++} in the platelet's response to membrane-insertion of the C5b-9 proteins is discussed both in the context of the known cellular effects of this ion and in the context of recent observations suggesting sublytic changes in platelet function after complement-mediated plasma membrane damage.

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IN ADDITION to their widely recognized cytolytic activity (reviewed by reference 1), there is now accumulating evidence that the C5b-9 proteins of the serum complement system also sublytically modulate normal cellular function through graded and reversible changes in the conductance properties of the plasma membrane of the target cell. For example, it has been reported that the binding of the C5b-9 proteins to human neutrophils can initiate the cell's respiratory response accompanied by a rapid rise in cytoplasmic free Ca^{++}, which occurs without subsequent lysis of the cell.[2-4] In other nucleated cells exposed to these proteins, evidence for a sublytic activiation of cellular phospholipases—leading to production of arachidonate and its metabolites—has also been presented.[5,6] Cell survival after membrane assembly of these potentially cytolytic proteins is likely to depend on the cell's capacity to actively maintain preexisting electrochemical gradients across the plasma membrane despite increased ion conductance mediated by the inserted C5b-9 pores.[7,8] In addition, restoration of membrane integrity may ultimately require the removal of functional C5b-9 complexes from the plasma membrane, for example, by exocytotic or endocytotic vesiculation of the bound proteins.[9,10]

We have recently demonstrated that the binding of the C5b-9 proteins to gel-filtered human platelets initiates a dose-dependent depolarization of the membrane potential (E^2_m) maintained across the plasma membrane of these anucleate blood cells.[11,12] We have also demonstrated that this electrochemical change can be fully reversed, E^2_m of the platelets spontaneously repolarizing to basal levels within minutes after membrane assembly of the C5b-9 complex. This reversible electrochemical change that is initiated upon C5b-9 binding was shown to occur in the absence of cell lysis and to depend on a functional plasma membrane Na/K-ATPase in the exposed platelets. Because a sublytic modulation of the electrochemical state of C5b-9–damaged platelets is likely to underlie the altered thrombotic reactivity of these cells observed after immunologic damage,[13,14] we undertook to identify the mechanism by which platelets restore their basal E_m in the face of the increased ion conductance that occurs after membrane insertion of the C5b-9 proteins. We report here that the active repolarization of electrochemical gradients subsequent to C5b-9 assembly on these cells occurs secondarily to a Ca^{++}-associated vesiculation of the plasma membrane, which serves to remove functional C5b-9 complexes and restore membrane integrity.

MATERIALS AND METHODS

Materials. DiS-C_3-(5) (3,3'-dipropylthiocarbocyanine iodide) was obtained from Molecular Probes (Junction City, Ore). Ouabain was from Sigma Chemical Co (St Louis). Sepharose CL-2B and Percoll were obtained from Pharmacia Fine Chemicals (Piscataway, NJ), and $Na^{125}I$ was from New England Nuclear (Boston). All other chemicals were of reagent or analytical grade.

Solutions. All solutions were freshly prepared using deionized H_2O obtained by reverse osmosis and ultrafiltration. Medium I: 145 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L sodium phosphate, 0.1 g/dL dextrose, 5 mmol/L PIPES, pH 6.8. Medium II: 145 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L sodium phosphate, 0.1 g/dL dextrose, 5 mmol/L HEPES, pH 7.4

Platelets. Platelet concentrates were obtained from normal adult volunteers by plasmapheresis, using either an IBM Model 2997 or Fenwall Model CS3000, and used within four hours of collection. Platelets were separated from plasma by centrifugation (20 minutes, 500g) and subsequent gel filtration on Sepharose CL-2B equilibrated in Medium I as previously described.[12] Platelet suspensions obtained by these methods were devoid of contaminating serum C7, C8, or C9 activity. Cell counting and resistive sizing were performed using Coulter Models ZBI and Channelizer (C 1000, Hialeah, Fla).

Complement proteins. Human complement proteins C5b6, C7, C8, and C9 were purified and assayed for functional activity as

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previously described.[15-18] Before addition to platelet suspensions, the proteins were dialyzed into Medium II and briefly warmed to room temperature.

C5b67 platelets. To assemble membrane-bound C5b67 complexes, gel-filtered platelets were suspended with C7 (5 μg per 1 × 10^8 cells) and 15 μg C5b6 added with rapid mixing in a final volume of 150 μL. After five minutes' incubation at room temperature, the C5b67 platelets were recovered and used immediately. For all experiments, matched-pair controls were prepared by omitting either C7 (C5b6 control) or both C5b6 and C7 (complement-free control).

Fluorescence measurements. Changes in membrane potential during C5b-9 assembly were monitored by use of the fluorescent indicator diS-C_3-(5) as previously described.[12] To 2 mL of Medium II in a cuvette equilibrated at 37 °C, 2.5 × 10^7 platelets (C5b67 or controls) were added. Two microliters diS-C_3-(5) were added from a 0.1-mg/mL stock in ethanol (final concentration, 1.87 × 10^{-7} mol/L) and allowed to equilibrate for three minutes with the stirred cell suspension. Excitation was at 622 nm (8 nm bandwidth) and emission at 670 nm (8 nm bandwidth). After equilibration of the dye, C5b-9 assembly was initiated by the addition of C8 and C9 (see figure legends). Steady-state fluorescence was monitored continuously throughout.

All fluorescence measurements were performed using an SLM 4800S equipped for sample stirring and temperature control and modified with a light tight port for sample injection. To correct for fluctuations in lamp output and instrument response, reference was routinely made to a quantum standard (rhodamine B in a sealed cuvette).

Calibration of diS-C_3-(5) fluorescence to E_m. Calibration of diS-C_3-(5) fluorescence to the platelet membrane potential was performed by methods previously described.[12]

Radiolabeling. C9 was radiolabeled with Na ^{125}I by the use of Enzymobeads (Bio-Rad Laboratories, Richmond, Calif) as previously described.[15] The specific activity was 0.18 mCi/mg.

Density-gradient analysis of C5b-9 platelets. To analyze the membrane distribution of bound C5b-9 complexes, C5b67 platelets were assembled as described earlier and gel-filtered on a Sepharose CL-2B column (30 × 0.9 cm) equilibrated in Medium I to remove unbound complement proteins. To 2.5 × 10^8 gel-filtered C5b67 platelets, 2.5 μg of C8 and 0.6 μg of ^{125}I-labeled C9 were added. Matched-pair controls were prepared by omitting C8. After incubation for 15 minutes at 37 °C, the samples were applied to discontinuous isotonic Percoll gradients made of 1 mL of 50%, 7 mL of 25%, and 5 mL of 7% Percoll in Medium II (omitting phosphate). The gradients were centrifuged in a Beckman J2-21 centrifuge for 30 minutes at 29,000 g, fractionated (0.4 mL per fraction), assayed for radioactivity and phosphorus, and prepared for electron microscopy.

Phosphorus assay. The distribution of platelet-dervied vesicles and membrane debris in Percoll gradients was estimated by quantitation of total phosphorus in gradient fractions, measured by the procedures of Bartlett.[19] Correction for a slight interference detected for Percoll solutions was accomplished by inclusion of equivalent amounts of Percoll in all phosphate standards.

Scanning electron microscopy. After fixation in 1% glutaraldehyde, samples were filtered through 0.4-μm pore diameter polycarbonate filters (Nucleopore, Pleasanton, Calif). The filters were dehydrated by successive incubation for ten minutes in 40%, 60%, 80%, and, finally, 100% ethanol. Critical-point drying from ethanol to liquid carbon dioxide was performed in a Tousimis SAMDRI-780 (Tousimis Research Corp, Rockville, Md). The samples were subsequently sputter coated with palladium gold (Hummer V, Technics, San Jose, Calif) and observed in a JEOL JSM-35C scanning electron microscope (JEOL, Ltd, Tokyo).

RESULTS

Evidence for C5b-9 pore inactivation. Membrane assembly of the C5b-9 complex on gel-filtered platelets has been shown to result in an initial depolarization of E_m that can be actively reversed, E_m repolarizing to its basal level of −60 mV in a sodium pump-dependent process.[12] Experiments performed in the presence of the Na/K-ATPase inhibitor ouabain suggest that this repolarization of the membrane potential after C5b-9 pore formation entails both ouabain-inhibitable and ouabain-insensitive components (Fig 1). As shown by the data of this figure, although ouabain pretreatment completely blocks the platelets' capacity to reestablish their initial electrochemical state (dashed trace), C5b-9 platelets exposed to ouabain after E_m has completely repolarized show only a small decrease in their membrane potential, which in part can be accounted for by the 5- to 12-mV electrogenic component of the sodium pump normally observed in metabolically active platelets (cf solid and dotted traces). These data therefore suggest that the response of target platelets to membrane insertion of the C5b-9 proteins includes both increased electrogenic Na^+ extrusion—partially compensating for the passive C5b-9 pore conductance—as well as inactivation of the membrane-inserted pores per se, restoring the net conductance of the plasma membrane toward that of control cells (cf dotted and dashed lines, Fig 1). In multiple experiments performed under conditions described for Fig 1, substantial variability was observed in the response to ouabain when added after repolarization of E_m to basal levels. This observed variability is likely to reflect preparation-to-preparation differences either in the initial metabolic state of the target platelets or in their capacity to inactivate the membrane-inserted C5b-9 proteins, restoring basal membrane ion conductances. Nev-

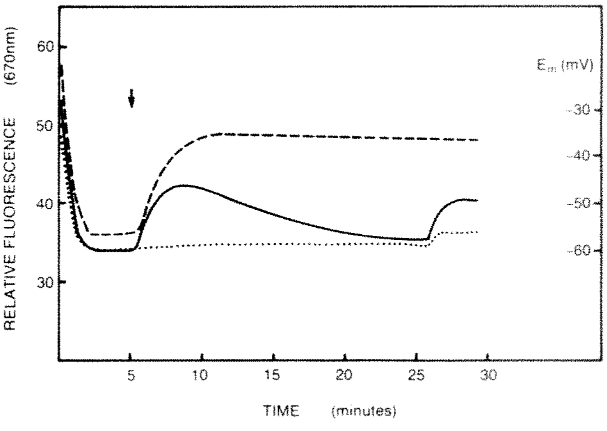

Fig 1. Reversible change in membrane potential after C5b-9 assembly: evidence for functional inactivation of membrane pores. C5b67 platelets suspended in Medium II (2.5 × 10^7 cells in 2 mL) were equilibrated with diS-C_3-(5) at 37 °C. At time indicated by arrow, C8 (0.35 μg) and C9 (1 μg) were added to the cuvette. Ouabain (final concentration, 50 μmol/L) was added at either time = 0 (dashed trace) or time = 26 minutes (solid trace). Fluorescence measurements (left ordinate) and calibration of membrane potential (right ordinate) were performed as described in Materials and Methods. Dotted trace indicates results for matched-pair control platelets exposed to C8 and C9 (see arrow) and then ouabain (at time = 26 minutes).

ertheless, in all experiments, the change in E_m measured upon addition of ouabain to C5b-9–treated platelets after E_m had returned to its initial value was always substantially less than the net depolarization measured for identical matched-pair samples pretreated with ouabain before C5b-9 assembly.

Plasma membrane vesiculation. Campbell and associates have recently proposed that neutrophils escape the cytolytic consequence of C5b-9 assembly by exocytotic vesiculation of the membrane-bound proteins, a process that they suggest may be related to the influx of Ca^{++} across the C5b-9–damaged plasma membrane.[3,4] Accordingly, we undertook to determine whether a similar mechanism (resulting in elimination of membrane-inserted complement pores) might account for the reversible changes in the electrochemical state of target platelets observed after C5b-9 assembly. Inspection of these cells by scanning electron microscopy after membrane assembly of C5b-9 proteins revealed marked vesiculation of the platelet surface (Fig 2), suggesting a C5b-9–induced plasma membrane shedding similar to that previously reported for C5b-9–treated nucleated cells.[3,4] Direct evidence for an association of the bound C5b-9 proteins with the small plasma membrane-derived vesicles was obtained by monitoring the distribution of ^{125}I-C9 and phospholipid (measured as inorganic phosphorus), after density fractionation of C5b-9–treated platelet membranes on Percoll gradients (Fig 3). As shown in Fig 3, density gradient centrifugation of C5b67 platelets after incubation with C8 plus ^{125}I-C9, resulted in two distinct peaks of radioactivity and phosphorus sedimenting into the Percoll gradient. Approximately 40% of the ^{125}I-C9 sedimented with a dense membrane fraction to near the bottom of the gradient (fractions 2 through 8), the remainder of the membrane-associated ^{125}I-C9 appearing as a diffuse band of lower density within the gradient (fractions 12 through 20). By contrast, results obtained for controls (C5b67 platelets incubated with ^{125}I-C9 in the absence of C8) revealed only a single small peak of membrane-associated radioactivity positioned near the bottom of the gradient, representing ^{125}I-C9 nonspecifically associated with the C5b67 cells (see below).

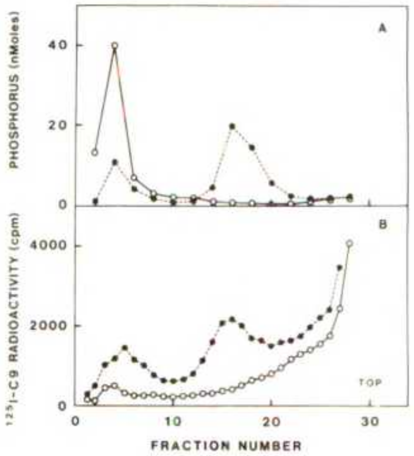

Fig 3. Density gradient centrifugation of C5b-9–treated platelets: separation of membrane vesicle fractions containing bound ^{125}I-C9. C5b67 platelets were prepared as described in Materials and Methods and then gel-filtered on Sepharose CL-2B to remove unbound complement proteins. After incubation (15 minutes, 37 °C) with ^{125}I-C9 (0.6 μg) and either 0 μg (open circles) or 2.5 μg (closed circles) C8 in Medium II, the platelet suspensions (2.5 × 10^8 platelets) were applied to an isotonic gradient of Percoll (0% to 50% wt/vol) in Medium II (omitting phosphate). After centrifugation (30 minutes, 29,000 g), 0.4-mL fractions were obtained, which were analyzed for phosphorus (panel A) and radioactivity (panel B). Visual inspection (not shown) of gradients before fractionation revealed separation of C5b-9–treated membranes (solid circles) into two diffuse bands (corresponding to fractions 2 through 8 and 12 through 20), whereas for controls (open circles), only a single dense band (corresponding to fractions 2 through 5) was evident.

In the absence of platelets, all of the ^{125}I-C9 remained at the top of the Percoll gradient when centrifuged under conditions of these experiments (see Materials and Methods).

Representative scanning electron micrographs obtained after fixation of these density-separated membrane fractions are shown in Fig 4. In the case of C8-negative control platelets, virtually all of the applied cells were detected in fractions 2 through 5, these cells morphologically appearing as partially activated platelets (Fig 4a). Remaining fractions of the gradient obtained for these controls were essentially

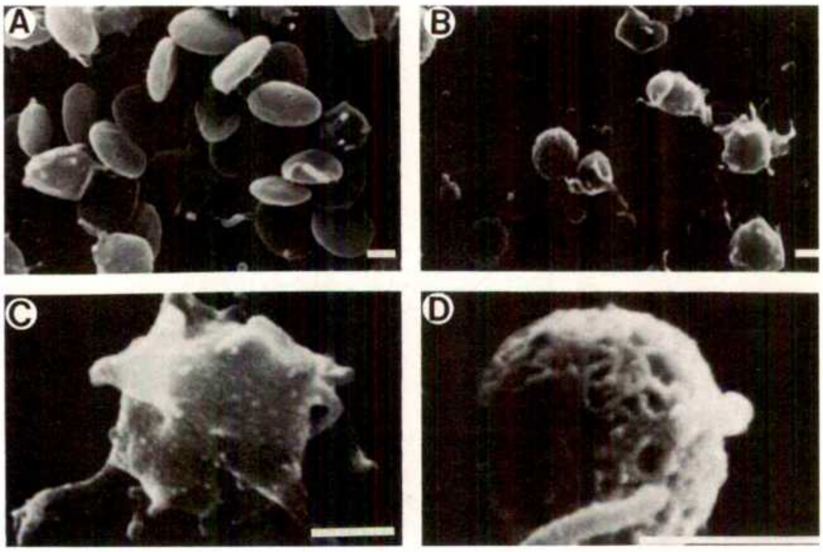

Fig 2. SEM of C5b-9 treated platelets. Controls (panel A) and C5b67 platelets (panels B through D) were prepared as described in Materials and Methods. After incubation (15 minutes, 37 °C) with C8 (0.35 μg) and C9 (1 μg), the cells were fixed with 1% glutaraldehyde and processed for scanning electron microscopy. Bar indicates 1 μm. Note vesiculation of membrane surface evident at higher magnification (panels C and D).

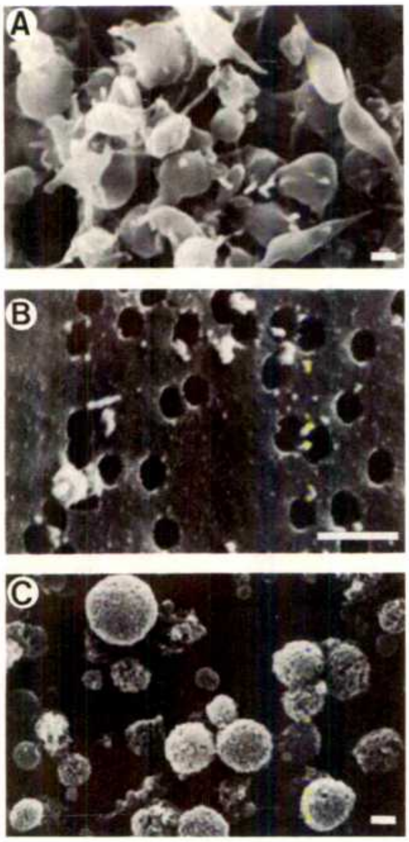

Fig 4. SEM of C5b-9 platelets after density gradient fractionation. Scanning electron microscopy of control (panel A) and C5b-9 platelets (panels B and C) after density gradient centrifugation (see Fig 3). Panel A shows fractions 2 through 5 of controls (open circles, Fig 3), while panel B (fractions 2 through 8, containing 33% of total applied phosphorus) and panel C (fractions 12 through 20, containing 45% of total applied phosphorus) were obtained from C5b-9 platelets (closed circles, Fig 3). Upper fractions (8 through 30) of control platelets were devoid of membrane material (not shown). Fixation and preparation for SEM performed as described in Materials and Methods. Bars indicate 1 μm.

devoid of phosphorus, and neither intact platelets nor vesicular material could be detected in these fractions by scanning electron microscopy. By contrast, in the case of density-separated C5b-9 platelets, inspection of fractions near the bottom of the gradient coinciding with the peak of phosphorus and ^{125}I-C9 detected in fractions 2 through 8 of Fig 3 revealed numerous small membrane vesicles of approximately 100 to 200 nm diameter, in addition to an occasional intact platelet (Fig 4b), while inspection of fractions 12 through 20 (representing the low-density membrane and radioactivity peak in the Percoll gradient; see Fig 3) revealed both large vesicles and numerous rounded platelets, which were markedly heterogeneous in size and displayed a prominently ruffled membrane surface with only occasional pseudopodia (Fig 4c). It is of interest to note that the surface features of the C5b-9 platelets collected in fractions 12 through 20 resemble that observed after incubation of normal human platelets with the calcium ionophore A23187.[20]

Role of external Ca^{++}. The marked vesiculation of the C5b-9 platelet surface that is associated with the release of bound ^{125}I-C9 and restoration of transmembrane electrochemical gradients suggests either (*a*) a fragmentation of the plasma membrane due to the insertion of these large amphiphilic proteins into the lipid bilayer, or (*b*) an induced response of the target cell, eg, triggered by the electrochemical changes accompanying formation of the C5b-9 pore. Because the results we have previously reported for C5b-9–treated platelets were obtained for experiments performed in the presence of external Ca^{++} (Figs 1 through 4 and reference 12), it was of interest to determine how the response of this cell to C5b-9 assembly is affected by the removal of this ion. As shown by the data of Fig 5, the addition of C8 and C9 to C5b67 platelets suspended in the presence of either 1 mmol/L Ca^{++} or 1 mmol/L EGTA results in an initial depolarization of E_m of the target cells. Although in the presence of 1 mmol/L external Ca^{++} this initial depolarization is immediately followed by a return of E_m to its basal level, in EGTA this subsequent repolarization of E_m is virtually eliminated, suggesting a requirement for Ca^{++} in the functional inactivation of the membrane-inserted C5b-9 pores. Density gradient fractionation of platelets after incubation with the C5b-9 proteins in the presence of EGTA revealed that the bulk of the membrane phosphorus and specifically bound ^{125}I-C9 radioactivity were present in fractions 12 through 20, coinciding with the low-density (large platelet) fraction recovered after C5b-9 treatment in the presence of 1 mmol/L external Ca^{++}. By contrast to the highly vesiculated and heterogeneous size observed for this low-density cell fraction after treatment with C5b-9 plus Ca^{++}, inspection of fractions 12 through 20 derived from C5b-9 plus EGTA-treated cells revealed a uniformly spherical cell population (averaging 2 to 4 μm diameter), with only occasional evidence of membrane vesiculation (cf Figs 4c and 6c). Also by contrast to results obtained for C5b-9–treated cells suspended in 1 mmol/L Ca^{++}, after C5b-9 treatment in EGTA only small amounts

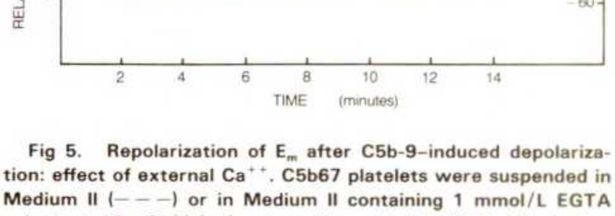

Fig 5. Repolarization of E_m after C5b-9–induced depolarization: effect of external Ca^{++}. C5b67 platelets were suspended in Medium II (— — —) or in Medium II containing 1 mmol/L EGTA substituted for Ca^{++} (---) and equilibrated with diS-C_3-(5). At time indicated by arrow, C8 (0.7 μg) and C9 (1 μg) were added. Fluorescence measurement and estimation of E_m performed as described for Fig 1. Results for control platelets in Medium II (· · · · · ·) and in Medium II containing 1 mmol/L EGTA (— - — - — - —) are also shown.

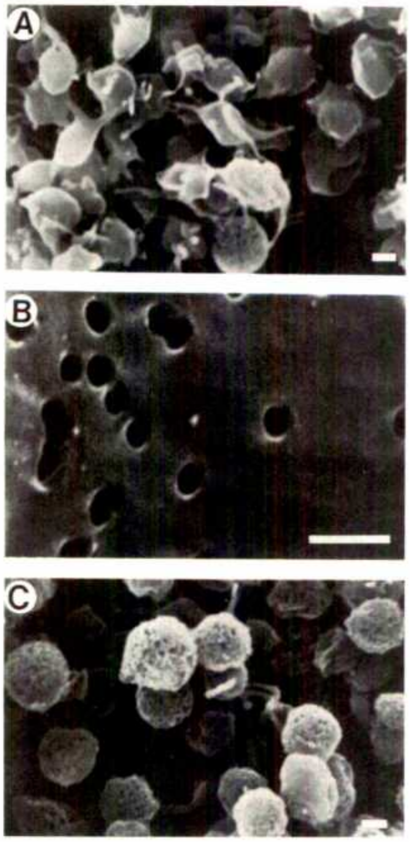

Fig 6. SEM of platelets treated with C5b-9 in absence of external Ca^{++}. C5b-9 platelets (and C8-negative controls) were prepared for Percoll density gradient centrifugation and scanning electron microscopy as previously described (see Figs 3 and 4), except that 1 mmol/L EGTA substituted for Ca^{++} in Medium II. Visual inspection (not shown) of gradients before fractionation revealed that control platelets sedimented to a single discrete band (corresponding to fractions 2 through 5; see Fig 3) and that C5b-9 platelets sedimented to a single diffuse band (coresponding to fractions 12 through 20). No lower (dense vesicle) band of C5b-9 membranes was evident (cf results for incubation in Ca^{++} medium, Figs 3 and 4) Panel A, SEM of fractions 2 through 5 (control platelets). Panels B and C, SEM of C5b-9 platelets prepared from fractions 2 through 8 (containing 13% of total applied phosphorus) and fractions 12 through 20 (containing 66% of total applied phosphorus), respectively. Bars indicate 1 μm.

of membrane material (as detected by phosphorus) were distributed in the remaining fractions of the density gradient (Fig 7), and repeated inspection by SEM demonstrated a marked reduction in the number of vesicles in the dense membrane fraction (corresponding to membrane material detected in fractions 2 through 8 of Fig 3 (cf Panel b of Figs 4 and 6). The reduction of membrane material plus ^{125}I-C9 sedimenting to the bottom of the density gradient after treatment in EGTA is consistent with the reduction in plasma membrane vesiculation previously noted in SEM of C5b-9 cells suspended in EGTA *v* Ca^{++}-containing media.

DISCUSSION

Although our results suggest that $>10^{-7}$ mol/L external Ca^{++} is required for (a) restoration of the platelet's basal E$_m$ after C5b-9–induced depolarization and (b) plasma membrane shedding of the inserted C5b-9 proteins, it nevertheless remains unresolved how this ion contributes to the vesiculation of C5b-9 complexes from the plasma membrane, leading to the restoration of transmembrane electrochemical gradients. Recent data reported by Campbell and associates[2-4] demonstrate that C5b-9 binding to erythrocyte and neutrophil membranes can result in a rapid increase in cytoplasmic Ca^{++} by diffusional influx across the plasma membrane. One might speculate that the membrane changes we observe after C5b-9 binding to platelets are directly related to this rise in cytoplasmic Ca^{++}, analogous to cellular changes observed in response to various externally added Ca^{++}-selective ionophores. For example, treatment of platelets with the ionophore A23187 has been shown to activate a Ca^{++}-dependent cytoplasmic protease that catalyzes the breakdown of actin-binding protein, thereby disrupting cytoskeletal organization.[21,22] It is conceivable that this Ca^{++}-induced degradation of the cytoskeleton is responsible both for the shape changes we observe after C5b-9 binding as well as for the shedding of membrane components, for example, by detachment of the cytoskeleton from the interior of the plasma membrane.[22] Alternatively, one might speculate that Ca^{++}-associated shedding of membrane-inserted C5b-9 components from the plasma membrane is related to the interaction of this ion with negatively charged phospholipids distributed on the inner surface of the lipid bilayer.[23,24] Evidence for transbilayer rearrangement of platelet membrane phospholipid ("flip-flop") associated both with a rise in cytoplasmic Ca^{++} and the degradation of cytoskeletal proteins has recently been discussed by Zwaal et al.[22]

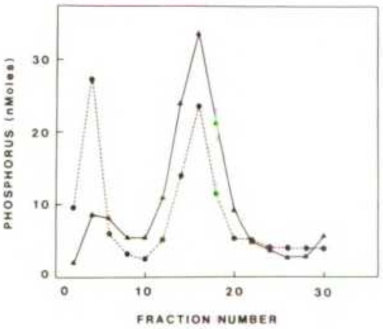

Fig 7. Inhibition of C5b-9–induced vesiculation by EGTA. Density gradient fractions obtained after C5b-9 assembly on gel-filtered platelets suspended in presence of either 1 mmol/L Ca^{++} (●- - -●) or 1 mmol/L EGTA (▲—▲). Percoll density separation was performed as described in Fig 3 and membrane phosphorus measured as described in Materials and Methods.

It is of interest to consider how the membrane vesiculation we observe after C5b-9 binding is related to the platelet-derived vesicles ("platelet dust") observed after thrombin stimulation of these cells,[25] as well as in association with intravascular platelet destruction initiated by the binding of platelet-reactive IgG.[26] For example, one might speculate that the diffusional influx of Ca^{++} represents a common pathway leading to cytoskeletal degradation and membrane vesiculation either after thrombin stimulation or after immune attack. Alternatively, the possibility that the C5b-9 proteins directly participate in the membrane changes observed after thrombin stimulation of this cell can be suggested on the basis of recent evidence that platelet-bound

C5b-9 complexes are normally generated from plasma during physiological clot formation in vivo and during thrombin-induced platelet aggregation in vitro.[5,13,14] A possible role for these cytolytic serum proteins in intravascular platelet activation has also been considered.[5,12]

Whether our data suggesting a Ca^{++}-dependent shedding of C5b-9 pores from the platelet surface (and consequent restoration of the cell's membrane potential) has significance for the ultimate function and survival of the immune-damaged platelet in the circulation remains unresolved. Based on the marked morphological changes in these cells observed under the conditions of our experiments, it seems unlikely that these C5b-9–damaged platelets would either persist in the circulation or exhibit normal hemostatic function. Nevertheless, one might speculate that plasma membrane changes associated with the vesiculation of the C5b-9 proteins—including changes in the transbilayer distribution of membrane phospholipid—would alter the hemostatic regulation of the clotting and fibrinolytic pathways on the platelet surface, possibly with thrombotic consequence. In this context, it is of interest to note that we[27] have recently demonstrated a marked increase in platelet prothrombinase activity after C5b-9 binding to the plasma membrane, which can be triggered in the absence of cell lysis.[28] In addition to potential interactions with the plasma clotting system, the possibility for cellular release of thromboxane A_2 and other thrombogenic agents due to activation of the arachidonate transformation pathway on C5b-9–induced Ca^{++} influx can also be considered.[5]

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Pretreatment Hematocrit as an Independent Prognostic Variable in Hodgkin's Disease

By Howard S. Jaffe, Edwin C. Cadman, Lenard R. Farber, and Joseph R. Bertino

Pretreatment hematocrit in 117 advanced-stage Hodgkin's disease patients treated with a combined modality therapy program was evaluated as an independent prognostic variable with regard to survival and relapse-free survival. Age greater than 40 years, and multiple extranodal sites of involvement were found to be statistically significant independent negative prognostic factors with regard to survival. Pretreatment hematocrit, however, was not an independent negative prognostic variable.
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ANEMIA as a prognostic factor in advanced Hodgkin's disease has been commented upon by many groups. Prior to the advent of recent treatment regimens, Bethell et al,[1] reporting on a series of 94 cases, noted that patients with any significant degree of anemia at the time of first presentation survived for a shorter period than those without anemia. Survival following diagnosis, for those with a hemogloblin level >11.0 g/dL (56 cases) was 67.9% at one year, 32.1% at three years, and 23.2% at five years. For those patients with levels of 11 g/dL or less, survival was 45.0, 18.5, and 8.0%, respectively. Roentgen therapy alone was used in all but five cases; nitrogen mustard was added late in the course of those five. Levinson et al[2] examined the packed red cell volume in 46 patients before therapy; mild anemia was arbitrarily defined as 3.8×10^6 to 4.2×10^6 packed red blood cells (RBC) per mm^3 of blood in males and 3.5×10^6 to 4.0×10^6 in females. Values less than these were termed moderate anemia. Of the 46, 24 patients were mildly anemic, and 10 moderately anemic. In this series, average survival time was 7 months or less following the appearance of moderate anemia before or after therapy. Median survival time from the onset of illness for the entire group was three and one-half years. Westling[3] studied 250 Hodgkin's patients, one-third of whom were anemic (hemoglobin <75% of normal for males, and <70% for females) on admission. Survival analysis showed significant differences between the anemic and non-anemic groups; 25% and 18% survival in the anemic group at five and ten years, compared with 47% and 29% survival in the normal group. More recently, Tubiana et al[4] reviewed the initial red cell number/mm^3 of blood in 354 patients with Hodgkin's disease. Anemia was defined as $<3.5 \times 10^6$ RBC/mm^3. Survival analyses confirmed Westling's data; of 61 patients in the anemic group, three- and five-year survival were 30% and 15%, compared to 48% and 30% in the nonanemic group.

The above studies and a host of others,[5-7] although uniformly portraying anemia as an unfavorable prognostic factor, must all be interpreted in the most cautious way. Aside from the obvious progression in diagnostics and therapeutics available to today's Hodgkin's disease patient, each of the above studies fails to examine anemia as an independent variable. As Kaplan[8] pointed out, "anemia is not an independent variable (in these studies), but is linked to generalized disease. It is therefore likely that the poor prognosis of these patients is primarily a reflection of the anatomic extent of their disease. There is no documentary evidence of the prognostic influence of anemia per se within a given clinical stage."

MATERIALS AND METHODS

The data for this study were generated as part of a larger study, described elsewhere in detail.[9] In brief, the study population was a series of 117 patients with advanced Hodgkin's disease treated with combined modality therapy at Yale University between 1969 and 1977. Each individual gave their informed consent to participate in this treatment program in accordance with the institutional guidelines of the Yale School of Medicine. All patients were evaluated per protocol as described.[9] Staging was done in accordance with the Ann Arbor classification system.[10] Complete blood counts were obtained upon entry. Newly diagnosed cases with stage IIIB or IV disease as well as those who had suffered relapses subsequent to curative attempts with radical radiotherapy were eligible. No patient who had previously received chemotherapy was included.

Remission status was assessed after three cycles (6 months of therapy) of MVVPP combination chemotherapy which included nitrogen mustard, vincristine, vinblastine, procarbazine, and prednisone. Complete remission was defined as the absence of all clinical, radiographic, and laboratory evidence of disease. Repeat biopsy examinations of accessible sites involved prior to the commencement of chemotherapy were performed. Partial responders were classified with nonresponders as induction failures.

Patients achieving clinical remissions received radiotherapy to all areas known to be involved with disease prior to chemotherapy. Radiotherapy was not administered prophylactically to adjacent, clinically uninvolved sites. Treatment was performed with either a 4 MeV or 6 MeV linear accelerator or cobalt-60 machine as described elsewhere.[9]

On completing radiotherapy, two additional cycles of chemotherapy were administered. All treatment was halted at the end of the fifth cycle (total treatment time 14 to 18 months). Remission status was assessed via annual reevaluation with appropriate radiographic and, when possible, tissue studies.

Minimum follow-up time was 42 months. Survival time was calculated from the onset of chemotherapy. Statistical significance of differences between various subgroups was evaluated with the chi-square test and the Hanky-Myers method.[11] Cox regression analysis was employed to evaluate the independent influence of different prognostic variables.[12]

RESULTS

Of 117 patients studied, 100 (85%) entered complete remission; there were 17 induction failures. Of the 100

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complete responders, 83 have remained continually free of Hodgkin's disease for periods of five to 14 years. Fifteen patients have experienced relapses. Two additional patients developed acute myelogenous leukemia while still in remission from Hodgkin's disease. Other complications are discussed in detail in the 1980 study.[9]

The complete remission rate for patients without "B" symptoms was 93% as compared to 81% for those with "B" symptoms ($P > .005$). Otherwise, there was no correlation between stage and response to therapy (Table 1). There was no correlation between pretreatment hematocrit and response to therapy within a given stage.

The rate of complete remission for patients under 40 years of age (90%) was significantly greater than the rate for those over 40 (61%) ($P > .001$). There was no correlation between pretreatment hematocrit and response to therapy within a given age bracket (Table 2).

The site of extranodal involvement *v* response to therapy is presented in Table 3. There was no significant difference between response in patients with one site of involvement versus those with nodal involvement only. However, response in those with multiple extranodal sites was only 53%, significantly ($P = .001$) decreased when compared with nodal or single-site involvement. There was no correlation between pretreatment hematocrit and response to therapy within a given subgroup.

Fifteen patients relapsed after achieving complete remission. There was also no statistical correlation between pretreatment hematocrit and relapse following complete remission (Table 4).

The five-year actuarial survival rate was 80% for the entire series, complete responders having a survival rate of 95%. Induction failures had a median survival time of one year. Relapse-free survival was 71% at five years.

The Cox regression model[12] was employed to analyze the independent influence of various patient characteristics on survival. Age >40 years and the existence of multiple extranodal sites of involvement were significant negative survival determinants. No other factor, including pretreatment hematocrit, was significant.

Table 1. Stage of Hodgkin's Disease *v* Response to Combined Modality Treatment

Stage	Number of Patients	Complete Remission		Mean Pretreatment Hct	
		No.	%	CR	IF
IIIB	21	19	90	35	33
IVA	7	7	100	39	—
IVB	29	23	79	36	36
RIIA	7	7	100	42	—
RIIIA	13	13	100	34	—
RIIIB	8	7	88	34	41
RIVA	17	14	82	37	33
RIVB	15	10	67	33	37
Total					
A Patients	44	41	93	38	33
B Patients	73	59	81	36	36
	117	100	85	37	36

Hct, hematocrit; CR, complete responder; IF, induction failure; R, previous radiotherapy.

Table 2. Age of Advanced Stage Hodgkin's Disease Patients *v* Response to Combined Modality Treatment

Age (y)	Number of Patients	Complete Remission		Mean Pretreatment Hct (vol %)	
		No.	%	CR	IF
<20	26	23	88	36	36
20 to 39	73	66	90	37	36
≥40	18	11	61	34	35
Total	117	100	85	37	36

Hct, hematocrit; CR, complete responder; IF, induction failure.

Table 3. Site of Extranodal Involvement *v* Response to Combined Modality Treatment and Relapse in Advanced Stage Hodgkin's Disease

	Number of Patients	Complete Remission		Relapse		Mean Pretreatment Hct (vol %)		
		No.	%	No.	%	CR	IF	R
Single Sites								
Bone Marrow	4	3	75	1	33	36	29	29
Lung	22	19	86	3	16	36	36	34
Liver	6	5	83	1	20	37	37	37
Bone	14	13	93	3	23	35	37	34
Miscellaneous	5	4	80	1	25	41	35	47
Total single	51	44	86	9	20	36	35	35
Multiple Sites	17	9	53	1	11	36	35	36
Total	68	53	78	10	19	36	35	35
All Bone Marrow	10	5	50	1	20	35	33	29
Only site	4	3	75	1	33	36	29	29
Additional sites	6	2	33	0	0	34	35	29

Hct, hematocrit; CR, complete response; IF, induction failure; R, relapse.

DISCUSSION

With the employment of combination and combined modality treatment programs, even those with advanced

Table 4. Pretreatment Hematocrit Versus Response to Combined Modality Treatment and Relapse in Advanced Hodgkin's Disease

Hematocrit	Number of Patients	Complete Remission		Relapse	
		No.	%	No.	%
28 to 30	12	10	83	3	30
31 to 33	17	16	94	3	19
34 to 36	33	26	79	4	15
27 to 40	36	30	83	3	10
≥41	19	18	95	2	11
Total	117	100	85	15	15

Hodgkin's disease can anticipate a high degree of complete remission (75% to 84%) and a five-year disease-free survival rate of about 70%.[9,13] Since our study only included Stage III B and IV A & B patients, correlation among pretreatment hematocrit and stage cannot be made. However, this would probably not affect our conclusion that pretreatment hematocrit is, in itself, not a statistically significant prognostic factor with regard to survival in patients with advanced Hodgkin's disease treated with a combined modality therapeutic regimen.

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Inhibition of Collagen-Induced Platelet Activation by 5′-*p*-Fluorosulfonylbenzoyl Adenosine: Evidence for an Adenosine Diphosphate Requirement and Synergistic Influence of Prostaglandin Endoperoxides

By Robert W. Colman, William R. Figures, L. Marie Scearce, Anne M. Strimpler, Fengxin Zhou, and A. Koneti Rao

The relative roles of platelet autacoids such as adenosine diphosphate (ADP), prostaglandin endoperoxides, and thromboxane A_2(TXA_2) in collagen-induced platelet activation are not fully understood. We reexamined this relationship using the ADP affinity analogue, 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA), which covalently modifies a receptor for ADP on the platelet surface, thereby inhibiting ADP-induced platelet activation. Collagen-induced shape change, aggregation, and fibrinogen binding were each fully inhibited under conditions in which FSBA is covalently incorporated and could not be overcome by raising the collagen used to supramaximal concentrations. In contrast, TXA_2 synthesis stimulated by collagen under conditions that produced maximum aggregation was only minimally inhibited by FSBA. Since covalent incorporation of FSBA has been previously shown to specifically inhibit ADP-induced activation of platelets, the present study supports the contention that ADP is required for collagen-induced platelet activation. Under similar conditions, indomethacin, an inhibitor of cyclooxygenase, inhibited collagen-induced shape change, indicating that endoperoxides and/or TXA_2 also play a role in this response. Shape change induced by low concentrations (10 nmol/L) of the stable prostaglandin endoperoxide, azo-PGH_2, was also inhibited by FSBA. These observations indicate a role for ADP in responses elicited by low concentrations of endoperoxides. However, at higher concentrations of azo-PGH_2 (100 nmol/L), inhibition by FSBA could be overcome. Thus, the effect of collagen apparently has an absolute requirement for ADP for aggregation and fibrinogen binding and for both ADP and prostaglandins for shape change. Aggregation and fibrinogen binding induced by prostaglandin endoperoxides also require ADP as a mediator, but ADP is not absolutely required at high endoperoxide concentration to induce shape change.

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FOLLOWING INJURY to endothelial cells and exposure of subendothelium, blood platelets adhere to the newly exposed collagen and undergo a phenomenon called shape change.[1] Shape change consists of a disk-to-sphere transformation and the extrusion of long, thin pseudopodia. Adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2) released by the adherent platelets recruit additional platelets to form aggregates.[2] The aggregated platelets then secrete part of the contents of their granules and together with fibrin form the tightly fused mass known as the hemostatic plug.

Since the identification of collagen as the active component of the connective tissue involved in hemostasis was made by Zucker and Borrelli,[3] numerous investigators have attempted to decipher the nature of the collagen-platelet interaction. Many studies have focused on the structural requirements of the collagen molecule to stimulate platelet aggregation and the release reaction,[4-7] and at least one study has focused on shape change,[8] the most proximate activation event. Most investigators have concluded that the quarternary structure of collagen is critical for the platelet-collagen interaction. This study focuses on another important aspect of collagen-induced activation, the nature of the intercellular and intracellular mediators such as released ADP, newly synthesized prostanoid derivatives. These mediators have been implicated in the cellular responses of activation, including shape change[9-13] and the exposure of fibrinogen binding sites.[14] The recent demonstration in our laboratories that ADP is required for aggregation[15,16] but not shape change[15] mediated by cyclic endoperoxides/TXA_2 resulted in a reexamination of the mechanism by which collagen may activate platelets.

5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA) has previously been used to explore many ADP-dependent interactions.[17] FSBA does not inhibit all ADP-dependent reactions; for instance, it has no effect on ADP-induced inhibition of adenylate cyclase in platelets prestimulated by prostaglandin E_1 (PGE_1).[18] However, its suitability for the study of platelet activation mechanisms rests on the following lines of evidence. FSBA covalently modifies a single polypeptide (molecular weight [mol wt] = 100,000) on the membrane surface of intact platelets.[19] Preceded by an electrophilic substitution and resulting in sulfonylbenzoyl adenosine (SBA)-labeled protein, this covalent incorporation parallels the inhibition of ADP-induced platelet shape change,[18,19] platelet aggregation,[20] and fibrinogen binding site exposure.[20] A related affinity analogue with the purine base guanosine substituted for adenosine, 5′-*p*-fluorosulfonylbenzoyl guanosine (FSBG), has no effect on ADP-induced shape change.[18] Further, FSBA inhibition of prostaglandin endoperoxide-induced aggregation parallels the activity of the ADP scavenger system—apyrase and creatinine phosphate/creatinine phosphokinase.[15] Thus, the affinity analogue FSBA is an appropriate probe to further investigate the role of ADP in collagen-induced platelet activation.

MATERIALS AND METHODS

Materials. FSBA was prepared by the method of Colman et al.[17] The prostaglandin endoperoxide analogue, 9,11-azo-PGH_2, was

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obtained from Dr K.C. Nicolau (Department of Chemistry, University of Pennsylvania, Philadelphia). Equine collagen fibrils were purchased from Hormon-Chemie, Munich. Human fibrinogen was purchased from Kabivitrum, Stockholm. ^{125}I was purchased from New England Nuclear, Boston. Bovine serum albumin, indomethacin, and calf intestinal adenosine deaminase were purchased from Sigma Chemical Co, St Louis. All other reagents were of reagent grade or better.

Platelet isolation. Platelet-rich plasma (PRP) 4 to 6 × 10^8platelets/mL was prepared by differential centrifugation of fresh whole human blood (120 g, 20 minutes, 23 °C). Volunteers claimed to have abstained from taking aspirin for 2 weeks prior to donation. Informed written consent was obtained. The blood was drawn into a 3.8% sodium citrate solution at a ratio of 5 mL anticoagulant per 45 mL blood. Suspensions of washed platelets were prepared by the method of Mustard et al.[21] After the final wash, the platelets were resuspended in a medium containing Na^+ (130 mmol/L), K^+ (2.7 mmol/L), Mg^{2+} (2 mmol/L), Cl^- (130 mmol/L), HCO_3^- (12 mmol/L), PO_4^{3-}(1 mmol/L), HEPES (10 mmol/L), glucose (560 mmol/L), and bovine serum albumin (3.5 mg/mL), at pH 7.4 and a platelet count of 5 to 8 × 10^8/mL. For TXA_2 synthesis experiments, platelets were washed by the method of Mills et al.[18] Calcium (2 mmol/L) was added for the fibrinogen-binding experiments.

Platelet aggregation and shape change. Aggregation was measured in an aggregometer (Chronolog, Havertown, Pa). The change in optical density was recorded on a chart recorder (LKB, Stockholm); the rate of aggregation was expressed in centimeters per minute. The instrument was calibrated at 100 mV for differential light transmission between the platelet suspension and the platelet suspension medium. The platelet suspension medium was platelet-poor plasma (PPP) for experiments conducted in PRP and Tyrode's buffer for those conducted in gel-filtered platelets (GFP). The platelet suspensions in these experiments were all stirred at 37 °C.

Shape change was measured on gel-filtered platelets diluted with an equal volume of 5 mmol/L EDTA to prevent aggregation, using a Chronolog lumiaggregometer at 37 °C. The rate of change in optical density was recorded on either an LKB recorder or a Fisher recorder and expressed as distance of pen deflection in millimeters per second after addition of an agonist. The platelet suspensions were 0.5 to 1.0 × 10^8 cells/mL in the cuvette.

Since FSBA potentially could form adenosine on incubation ($\leq$.05 mol adenosine per mole FSBA), all aggregation and shape change experiments were performed in the presence of adenosine deaminase at a concentration of 2 U/mL. Inclusion of this enzyme blocked the immediate inhibitory effects[18] by FSBA (100 μmol/L) or adenosine (400 μmol/L).

Platelet modification by FSBA. FSBA was dissolved in dimethyl formamide (DMF) to a high enough concentration so that the final concentration of DMF added to the platelets was less than 2.5%. Adenosine deaminase (final concentration, 2 U/mL) was first added to the platelet suspensions of 2.4 × 10^8 platelets/mL prior to the addition of the FSBA-DMF solution. The mixture was incubated for 20 minutes at 37 °C. DMF in equivalent amounts was added to all control samples. At the conclusion of the incubation, the platelets were sedimented (11,100 g, 20 minutes, 23 °C) and resuspended in fresh Tyrode's buffer. To ensure that the platelets were refractory to ADP, we challenged an aliquot of the platelets with 10 μmol/L ADP and measured aggregation and shape change. Only platelets that failed to aggregate or undergo shape change were used. Concentrations of FSBA required to produce complete refractoriness in different donor platelets varied from 40 to 100 μmol/L, and the time at which complete inhibition of shape change occurred varied from 15 to 60 minutes. Each experiment represents trials performed in duplicate from the platelets of a single donor and is representative of at least three separate donors.

Preparation of ^{125}I-fibrinogen. Human fibrinogen radiolabeled with ^{125}I was prepared as described by Figures et al.[20]

Fibrinogen binding to platelets. Specific binding of ^{125}I-fibrinogen to platelets was carried out using a separation technique of centrifugation through silicone oil.[15,20] The range of ^{125}I-fibrinogen concentrations used (5 to 80 μg/mL) was chosen to saturate high-affinity binding sites on the platelet surface. Incubation mixtures containing ^{125}I-fibrinogen, EDTA, collagen, and FSBA were layered on silicone oil in microcentrifuge tubes, and the platelets were sedimented through the oil layer at 12,000 g for three minutes. The tips of the centrifuge tubes containing the platelet pellet were severed from the rest of the tubes and were counted in a gamma counter (Intertechnique, Baltimore) to yield the total amount of fibrinogen bound. Nonspecific binding of fibrinogen to the platelet surface was determined by adding 2 mmol/L EDTA to the incubation mixture to inhibit collagen-induced exposure by fibrinogen binding sites. Counts associated with the platelet pellet under these conditions were considered to be the result of nonspecific fibrinogen binding. Specific binding was calculated as the difference of total binding and nonspecific binding. Results presented here represent specific fibrinogen binding. All experiments were carried out at 37 °C.

Measurement of TXA_2. TXA_2 is an unstable compound and is rapidly converted into the more stable TXB_2.[2] In the present study, TXA_2 was determined by radioimmunoassay in its TXB_2 form. The assay was performed using the antiserum against TXB_2 as described by Lewy et al[22] and provided by Dr J.B. Smith (Thrombosis Research Center). Two experiments were performed; the samples were assayed in duplicate.

RESULTS

Effect of FSBA on the rate of platelet aggregation induced by collagen. Washed platelets were treated with either FSBA or an equivalent volume of DMF, the carrier solvent for FSBA, for various times prior to the addition of collagen, 4 μg/mL (Fig 1). DMF had no significant effect on the rate of aggregation by collagen. Platelets incubated with FSBA, however, exhibited an aggregation rate that progressively diminished over a 20-minute period.

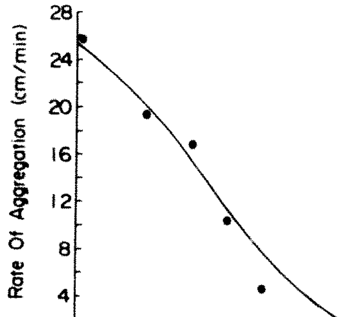

Fig 1. Time-dependent inhibition of collagen-mediated platelet aggregation by FSBA. Washed platelets were incubated with 40 μmol/L FSBA at 37 °C in the presence of adenosine deaminase (2 U/mL) after centrifugation and resuspensions (see Methods). At indicated times, a 0.5-mL aliquot was withdrawn and placed in an aggregometer. Collagen (2 μg) was then added, and the aggregation, as indicated by the deflection of the chart recorder pen, was measured. Inhibition of aggregation was complete by 13 minutes.

To ascertain whether the inhibition could be overcome by increasing the concentration of collagen, we incubated washed platelets with FSBA (Table 1) and demonstrated that no response occurred to 10 μmol/L ADP. Collagen produced a maximum response in platelets incubated with DMF at 4 μg/mL, and little further increase was noted with 40 mg/mL. FSBA totally inhibited collagen-induced aggregation at both 4 and 40 μg/mL.

Effect of FSBA on collagen-induced exposure of fibrinogen binding sites. Platelet aggregation was induced by 4 μg/mL collagen in the presence of concentrations of ^{125}I-fibrinogen consistent with binding of the protein to high-affinity sites (Fig 2). The platelets were incubated with FSBA (40 μmol/L) or DMF for 20 minutes. Fibrinogen bound to platelets incubated with DMF increased proportionally to the concentration of added fibrinogen. The fibrinogen bound to platelets incubated with FSBA was markedly diminished and was similar to that observed with EDTA, which inhibits specific binding of fibrinogen.

Effect of FSBA on TXA_2 formation stimulated by collagen in aggregating platelets. The possibility that FSBA could block any of the reactions leading to TXA_2 synthesis and thus inhibit platelet aggregation and exposure of fibrinogen binding sites by collagen was then investigated. In the absence of collagen but in the presence of the amount of calcium in HEPES-buffered Tyrode's solution (~50 μmol/L), stirred washed platelets preincubated with DMF contained TXA_2 at a concentration of 38 nmol/L and did not aggregate. Following incubation with collagen (4 μg/mL), the TXA_2 level rose to 260 nmol/L, coincident with maximum aggregation at two minutes. When platelets were preincubated with FSBA (100 μmol/L) for 20 minutes and stimulated with collagen (4 μg/mL), the concentration TXA_2 at two minutes was 170 nmol/L.

Effect of FSBA on the rate of platelet shape change induced by collagen. Washed platelets were incubated with FSBA (40 μmol/L) for various times prior to the addition of 4 μg/mL collagen (Fig 3). The velocity of shape change was measured in the presence of EDTA (2 mmol/L) to inhibit aggregation. A progressive inhibition of shape change was observed with a similar time course to that of aggregation (Fig 1). Platelets incubated with DMF, the solvent from FSBA, for the same time showed no inhibition of the velocity of shape change (not shown). The platelets were then incubated with various concentrations of FSBA for 20 minutes prior to measuring the velocity of shape change induced by collagen (Fig 4). A concentration-dependent inhibition was observed that reached a maximum of greater than 88% inhibition at concentrations between 25 and 40 μmol/L. The IC_{50} (concentration to produce 50% inhibition of maximum rate of shape change) was approxi-

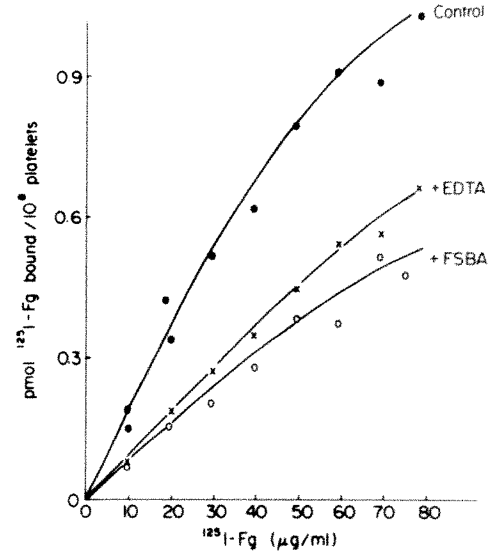

Fig. 2. Effect of FSBA on collagen-mediated fibrinogen binding. Washed platelets were preincubated with FSBA (40 μmol/L) as indicated in Fig. 1. These platelets were then incubated with collagen (4 μg/mL) and various concentrations of ^{125}I-fibrinogen for 20 minutes at 20 °C. Platelets incubated with DMF were also incubated separately with EDTA (2 mmol/L) for one minute prior to adding ^{125}I-fibrinogen. FSBA was able to reduce the fibrinogen binding to background levels as indicated by the labeling in the presence of EDTA.

Table 1. Effect of Increasing Collagen Concentrations on Inhibition of Platelet Aggregation by FSBA

Agonist	Concentration	Maximum Rate of Aggregation (cm/min)	
		DMF	FSBA
ADP	10 μmol/L	23.8	0
Collagen	4 μg/mL	24.0	0
	8 μg/mL	24.8	0
	16 μg/mL	29.0	0
	20 μg/mL	29.3	0
	40 μg/mL	28.1	0.5
Azo-PGH$_2$	1 μg/mL	20.3	0
Azo-PGH$_2$	2 μg/mL	20.8	0

Washed platelets were incubated with 100 μol/L FSBA in DMF or DMF alone for 30 minutes at 37 °C. ADP, collagen, or azo-PGH$_2$ at various concentrations were added and the maximum rate of aggregation recorded. Each point is the mean of duplicate determinations in three different donors.

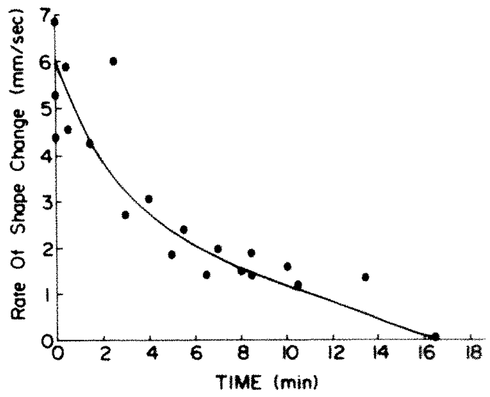

Fig 3. Time-dependent effect of FSBA on collagen-induced shape change. Washed platelets were incubated with 40 μmol/L FSBA as in Fig 1. At the indicated times, a 0.5-mL aliquot was placed in an aggregometer, and 4 μg collagen was added. Shape change was gradually inhibited over time, with complete inhibition observed at 16.5 minutes in this experiment.

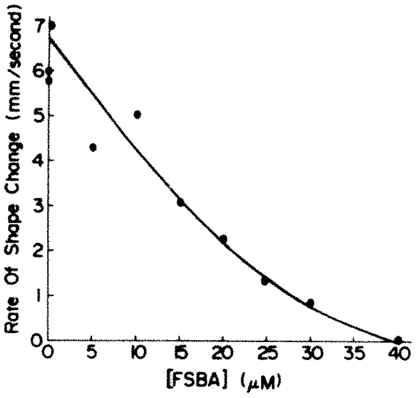

Fig 4. Concentration-dependent effect of FSBA on collagen-induced shape change. Washed platelets were incubated at 37 °C with various concentrations of FSBA for 40 minutes. At the end of the incubation, 0.5 mL was placed in an aggregometer, 4 μg collagen was added, and the rate of shape change as indicated by the deflection of the chart recorder was measured. Inhibition was virtualy complete at concentrations of 30 μmol/L or more.

mately 14 μmol/L FSBA, similar to that reported for inhibiting ADP-induced shape change.[19] The inhibition could not be reversed by concentrations of collagen as high as 40 μg/mL (Table 2).

Effect of indomethacin on the rate of collagen-induced shape change. At concentrations of indomethacin that inhibit cyclooxygenase (10 μmol/L), shape change was inhibited at collagen concentration from 4 to 40 μg/mL (Table 2), whereas ADP-induced shape change was not inhibited.

Effect of FSBA on Azo-PGH$_2$-induced platelet shape change and aggregation. Since collagen is known to cause both release of ADP and synthesis of prostaglandin endoperoxides, the effect of FSBA on shape change stimulated by endoperoxide was assessed. Washed platelets were incubated with FSBA or DMF for various times before the addition of azo-PGH$_2$ (10 nmol/L). In the presence of DMF, the cells changed shape at all incubation times. On incubation with FSBA, the platelet shape change with azo-PGH$_2$ was progressively inhibited until no shape change was observed at 60 minutes (Fig 5). However, increasing concentrations of azo-PGH$_2$ (up to 100 μmol/L) were able to overcome the inhibition of shape change by FSBA (Fig 6). In contrast, platelets preincubated with FSBA (100 μmol/L 20 min.) in the presence of adenosine deaminase and refractory to ADP failed to aggregate with even 1 μmol/L azo-PGH$_2$ (Table 1) in agreement with a previous report.[15]

Table 2. Effect of Increasing Concentrations of Collagen on Inhibition of Platelet Shape Change by FSBA

		Maximum Rate of Shape Change (mm/s)		
Agonist	Concentration	DMF	FSBA	Indomethacin
ADP				
(μmol/L)	10	2.9	0	2.65
Collagen				
(μg/mL)	4	6.4	0	0
	8	7.5	0	0
	16	8.25	0.1	0
	20	8.0	0.1	0
	40	8.0	0.2	0

Washed platelets were incubated with 100 μmol/L FSBA in DMF or DMF alone for 30 minutes or 10 μmol/L indomethacin for 30 seconds at 37 °C. ADP or collagen at various concentrations was added, and the maximum rate of shape change recorded. Each point is the mean of duplicate determinations.

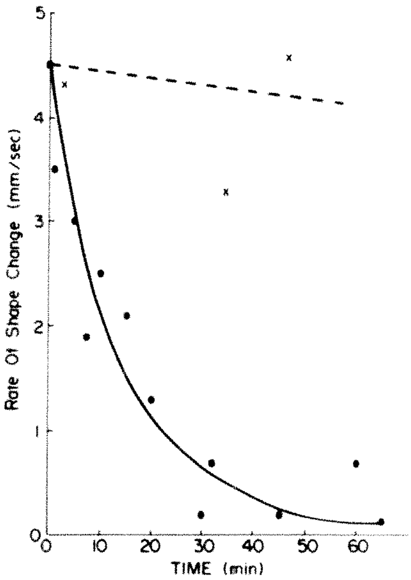

Fig 5. Time-dependent effects of FSBA on azo-PGH$_2$-induced shape change. Washed platelets were incubated with 40 μmol/L FSBA (●) or with DMF (×). Aliquots (0.5 mL) were removed at various times, and 10 nmol/L azo-PGH$_2$ was added in the aggregometer. After 60 minutes the azo-PGH$_2$-induced shape change was completely inhibited.

DISCUSSION

The data in this study emphasize the complexity of the interaction of agonists, intracellular autacoids, and intercel-

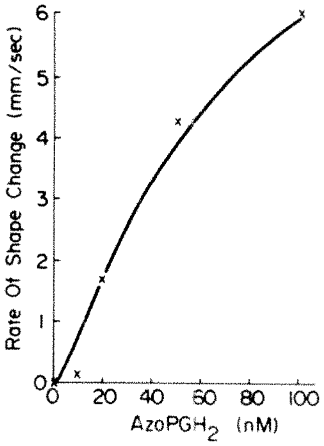

Fig 6. Concentration-dependent effects of azo-PGH$_2$-induced shape change on FSBA-labeled platelets. Washed platelets were incubated with 40 μmol/L FSBA at 37 °C. After 60 minutes, 0.5-mL aliquots were removed, and increasing concentrations of azo-PGH$_2$ were completely overcome by an azo-PGH$_2$ concentration at 100 nmol/L.

lular messengers in the responses of platelets to collagen. The collagen used is already in the form of multimers, and the structure or type of collagen is not an issue here. We have shown that covalent incorporation of SBA into platelets after incubation with FSBA renders the platelet unresponsive to the induction of aggregation and exposure of fibrinogen binding sites by ADP.[20] Thus, pretreatment with FSBA produces a time-dependent inhibition of collagen-induced platelet aggregation (Fig 1) and prevents collagen-induced fibrinogen binding sites (Fig 2); both effects are a function of the FSBA concentration. Since FSBA under the same conditions inhibits these same functions when stimulated by ADP, the results with collagen imply a role for ADP in those responses. FSBA also inhibits ADP-induced shape change.[18,19] The inhibition of collagen-induced shape change by FSBA in both a time- (Fig 3) and concentration- (Fig 4) dependent fashion implies that ADP is also required for collagen-induced shape change.

Previous studies of collagen-induced platelet aggregation have yielded controversial results as to whether ADP is required. Geratz et al[23] attributed the action of collagen on platelets entirely to ADP, whereas Kinlough-Rathbone et al[24] felt that ADP was only partly responsible. However, Nunn[25] concluded that ADP does not play an essential role in collagen-induced aggregation. One method used to arrive at the latter conclusion was the use of adenosine to inhibit ADP effects on platelets. High concentrations of collagen (20 to 30 μg/mL) were unaffected by adenosine. However, considerable evidence exists that suggests that adenosine inhibition of platelet function is mediated through a mechanism separate from ADP stimulation.[18] The characteristics of an adenosine receptor are distinct from those of any ADP receptors as evidenced by the different pattern of agonist and antagonist effects.[26] In general, the inhibitory effects of adenosine seem to be mediated by stimulating adenylate cyclase and increasing cyclic adenosine monophosphate levels,[27,28] and these effects can be enhanced by phosphodiesterase inhibitors.[29]

Nunn[25] also observed that the amount of ADP released by collagen at the onset of aggregation cannot alone account for the extent of aggregation. This observation supported the theory of Packham et al,[30,31] which attributed collagen-induced aggregation to the effects of released ADP and newly synthesized prostaglandin endoperoxides. By combining indomethacin (to block cyclooxygenase-catalyzed endoperoxide synthesis) and creatinine phosphate/creatinine phosphokinase (to convert ADP to adenosine triphosphate [ATP], an antagonist of ADP), Packham et al[30,31] completely inhibited collagen-induced platelet activation. Neither agent alone completely blocked platelet activation by high concentrations of collagen. FSBA completely inhibited aggregation even by high concentrations of collagen (Table 1). The difference from the results of Packham et al[30,31] may have been due to small concentrations of ADP that are not removed by the ADP-depleting enzymes. In contrast, FSBA inhibited TXA_2 formation induced by collagen only minimally, and its effects were mainly due to its ability to inhibit ADP binding.[18-20] Endoperoxide-induced platelet aggregation and fibrinogen binding was shown to require ADP by Morinelli et al,[15] and this was supported by the studies of Rao et al[16] using platelets with the storage pool deficiency. Thus, FSBA exerts its effect on collagen-induced platelet aggregation and fibrinogen binding by a direct effect on blocking an ADP receptor.

The effect of collagen on platelet shape change is more complex. After a short lag, both prostaglandin endoperoxides and TXA_2 are produced, and ADP is released after adding the collagen. Either FSBA or indomethacin can totally inhibit shape change even at very high collagen concentrations (Table 2). This suggests that collagen-induced shape change is dependent on a synergistic role of both these autocoids. In contrast, shape change induced by very high concentrations of azo-PGH_2 (100 nmol/L) could not be inhibited by FSBA,[15] implying a direct effect of TXA_2 and/or prostaglandin endoperoxides independent of ADP. However, in this study, we show that platelets activated by low concentrations of azo-PGH_2 (10 nmol/L) are inhibited by FSBA in a time-dependent manner (Fig 5). By increasing the concentrations of azo-PGH_2, FSBA inhibition was overcome (Fig 6) at a concentration of 100 nmol/L azo-PGH_2. It thus appears that at low concentrations of azo-PGH_2 potentiation of the response by ADP is necessary for induction of shape change.

It is interesting to note that collagen-induced TXB_2 synthesis was partially inhibited by FSBA. These preliminary findings suggest that secreted ADP may play a potentiating role in thromboxane synthesis as well. Such an explanation is supported by the observations of Siess et al[32] showing that ATP and enzyme systems that deplete ADP inhibit not only aggregation but also phospholipase C activation and arachidonic acid metabolism induced by endoperoxide analogues. The effects of FSBA on these pathways require further exploration.

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Acquired Thrombasthenia Due to GPIIb/IIIa-Specific Platelet Autoantibodies

By Herwig Niessner, Kenneth John Clemetson, Simon Panzer, Christian Mueller-Eckhardt, Sentot Santoso, and Peter Bettelheim

An otherwise healthy woman developed a hemorrhagic diathesis with fluctuating clinical symptoms and laboratory findings, but without thrombocytopenia, over 8 years. In periods of bad clinical condition, a platelet defect, characteristic of thrombasthenia, was found. In contrast to classic thrombasthenia, electrophoresis of the patient's platelet membranes revealed normal amounts of glycoproteins IIb_{α}, IIb_{β}, and IIIa in the normal positions. Monoclonal antibodies, specific for GPIIIa and GPIIb/IIIa, respectively, bound normally to the $\mathrm{P1}^{\mathrm{A1}}$-positive platelets from the patient. Although no antibody and no platelet function inhibitor were evident in the autologous plasma, an IgG1 antibody that was bound to the patient's platelets and was directed against GPIIb/IIIa could be demonstrated. After elution from the patient's platelets, this antibody immunoprecipitated GPIIb (both subunits), IIIa, and a 200-kilodalton (kd) band (probably undissociated GPIIb/IIIa complex) from solubilized normal platelets, but did not react with thrombasthenic platelets. Adding the eluate from the patient's platelets to normal platelet-rich plasma immediately caused concentration-dependent inhibition of adenosine diphosphate (ADP)-induced and collagen-induced aggregation and also strong inhibition of ADP-stimulated fibrinogen binding. Because it was very unlikely from the patient's medical history that the antibody was caused by alloimmunization, the hemorrhagic diathesis must be interpreted as acquired thrombasthenia due to an anti-GPIIb/IIIa autoantibody.

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GLANZMANN'S thrombasthenia (GT) is an autosomal recessive disorder resulting, in homozygotes, in a moderately severe bleeding tendency from early life.[1,2] Although the clinical severity varies somewhat among patients, the tendency to bleed remains more or less unchanged in the same patient. It is now well established that the underlying defect in GT is a deficiency of two platelet membrane glycoproteins, designated GPIIb and GPIIIa.[3,4] This membrane abnormality is closely related to the failure of primary platelet aggregation in GT. The lack of response to any concentration of ADP, collagen, or arachidonic acid, but normal agglutination by ristocetin, is characteristic of GT.[2]

The case we report also showed a platelet function defect characteristic of GT. In contrast to the inherited disorder, however, in our patient the symptoms developed during life and both clinical and laboratory findings fluctuated. Even in periods of evidently severe platelet function defects, GPIIb/IIIa were demonstrably normal. Because biochemical and immunological data definitely showed an antibody directed against GPIIb/IIIa, it seems justified to interpret the case as acquired GT due to anti-GPIIb/IIIa autoantibodies.

Unlike patients with chronic autoimmune thrombocytopenic purpura (ATP), in whom autoantibodies against GPIIb/IIIa were also recently found,[5-7] our patient had a normal platelet count throughout the observation period. It is likely that the antibodies are directed against different antigenic determinants on the GPIIb/IIIa complex, resulting in a GT-like functional defect without significant thrombocytolysis, whereas in ATP the epitope involved may be related to immunoinjury of platelets.

MATERIALS AND METHODS

Case Report

The patient, W, is a 42-year-old secretary. No cases with an increased bleeding tendency are known in her family. The father of the patient and her only son had normal platelet function. Prior to 1977, the patient's medical history was normal, and no increased bleeding tendency was observed. An appendectomy in 1964, three tooth extractions in 1971, and pregnancy and delivery in 1972 were uneventful. The patient had no history of either blood transfusions or any drugs at that time except for oral contraceptives from 1972 to 1979. In January 1978, she first noticed spontaneous ecchymosis and hematoma, or, with minor trauma, episodes of nosebleeds and menorrhagia. The patient was then seen for the first time in our hospital. Physical and general laboratory investigations gave normal results. No signs of an underlying autoimmune disorder such as systemic lupus erythematosus were found. No antinuclear antibodies (investigations also included the subsets anti-"Sm," anti-RNP, anti-SS-A, and anti-SS-B) were evident. HLA-typing revealed the following pattern: HLA-A24,26; B16,35; Cw4; DR 5,w6. The results of laboratory studies of hemostasis (on this and other occasions) are given in Table 1. Platelet membrane glycoproteins were also investigated by two-dimensional gel electrophoresis, and GPIIb/IIIa were normal.

During the following years, both clinical symptoms and laboratory findings (Table 1) fluctuated. For more than 1 year, the patient remained unchanged. Bleeding complications could be controlled by local measures, and no blood transfusions were necessary. After continuous improvement in 1979 and 1980, the patient became completely normal in 1981. In 1982 and 1983, the patient did well; interruption of a second pregnancy and tubal ligation by laparotomy (in 1982) were uneventful. In December 1983, however, a severe hemorrhagic diathesis again developed, with spontaneous large hematomas, epistaxis and, in particular, profuse menorrhagia, necessitating, for the first time, several blood transfusions.

Immunoglobulins (Sandoglobulin, Sandoz, Switzerland) were given intravenously (IV) in a dosage of 400 mg/kg body wt for five consecutive days in June 1984. Duke's bleeding time, platelet retention, and platelet aggregation (induced by ADP and collagen) were assayed on days 1, 5, and on days 12, 19, and 26. No significant

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Table 1. Platelet Function Investigations of Patient W

Platelet Function	1978 Jan 30	1979 Nov 13	1982 Sept 9	1983 Dec 22	1984 Jan 4	1984 June 6	1984 Nov 11	1985 June 20	Normal
Platelet count ($10^3/\mu L$)	223	214	295	226	182	220	195	210	150–350
Bleeding time (min)									
Duke's method	>10	7	3	>10	>10	>10	>10	2	<4
Borchgrevink technique	>20	16	6	>20	ND	ND	ND	5	<9
Clot retraction (%)	40	ND	80	36	ND	ND	40	90	>90
Platelet retention	11	21	97	34	14	14	12	91	>80
Platelet aggregation (ΔOD/t, mm)									
Collagen (3.0 μg/mL)	0	0	162	0	0	0	0	330	150–350
ADP (1.0 μmol/L)	0	ND	181	0	0	0	0	180	170–330
Arachidonic acid (1.0 mmol/L)	ND	ND	214	0	0	0	0	255	150–360
Ristocetin (1.7 mg/mL)	245	ND	254	270	ND	234	400	400	160–420

Max ΔOD/t, rate of platelet aggregation; ND, not done.

change in any of the laboratory parameters in comparison to pretreatment values was observed.

In November and December 1984, prednisolone was given orally: after therapy was initiated with 100 mg on day 1 and 2, the dose was reduced to 75 mg on days 3 and 4, and to 50 mg on days 5 and 6. This was followed by a gradual tapering of the dosage (5-mg reduction every second day) to a daily dose of 30 mg, maintained for 2 weeks. Subsequently, prednisolone was slowly reduced again (5-mg reduction every second day) and then discontinued after 36 days. A dose of ≥30 mg a day was taken by the patient for 26 days. Hemostatic tests (platelet count, Duke's bleeding time, platelet retention, ADP-induced and collagen-induced platelet aggregation) were performed weekly during and 1 and 2 weeks after prednisolone therapy. No significant changes were observed.

Neither treatment had any distinct clinical benefit, and no major side effects were observed. In particular, the tendency to spontaneous hematomas and to profuse menorrhagia remained unchanged. The patient again recovered spontaneously in 1985. The patient had agreed to participate in the therapeutic attempts and gave informed consent.

Methods

Coagulation data. Routine coagulation tests were performed by standardized methods.[8] Platelet factor 4 (PF4) and β-thromboglobulin (β-TG) levels were assessed by radioimmunoassays using commercially available kits (Amersham, England).

Platelet Investigations

Morphology. Platelet morphology was studied by light and transmission electron microscopy. Size distribution was analyzed by an impedance particle counter (CC 800, Sysmex, TOA, Japan).

Function. Bleeding time (slight modifications of the Duke and Borchgrevink methods), platelet retention (glass bead method), and clot retraction were performed by standardized routine methods.[9] Platelet aggregation was measured with citrated platelet-rich plasma (PRP) adjusted with platelet-poor plasma (PPP) to ~2.5×10^5 platelets per microliter; 0.4 mL of PRP were incubated for one minute at 37 °C (Fresenius aggregometer, FRG), and the following inducers were added (final concentrations): Collagen (1.5, 3, and 10 μg/mL, Hormonchemie, FRG); ADP (0.5, 1, and 2 μmol/L, Boehringer-Mannheim, FRG); arachidonic acid (0.5 and 1.0 mmol/L, Sigma); ristocetin (1.5 and 1.7 mg/mL, Lundbeck, Denmark); thrombin (0.1 U/mL, Behringwerke, FRG); and adrenaline (3 to 100 μmol/L, Hoechst, FRG). The rate of platelet aggregation (max ΔOD/t) was determined by the tangent to the steepest slope of the aggregation tracing.

ADP and ATP concentrations were measured by the firefly system[9] (Turner fluorometer, model 111, Palo Alto, Calif). Results were expressed in μmol/L per 10^{11} platelets.

Membrane glycoprotein composition. Binding of platelet-specific monoclonal antibodies (mab) directed against epitopes on major glycoproteins was studied by indirect immunofluorescence. Washed platelets were incubated with optimal dilutions of mabs from murine ascitic fluid, washed and stained with fluorescein-conjugated goat F(ab')$_2$ anti-mouse-IgG. Mouse ascites served as a negative control. The following mabs were used: AN 51 (anti-GP Ib),[10] J 15 (anti-GPIIb/IIIa),[11] and C 17 (anti-GPIIIa).[12]

For biochemical analysis, platelets from patient W, from patients with GT, and from normal individuals were surface-labeled by the periodate/^{3}H-NaBH$_4$ method),[13] solubilized, and run in one-dimensional and two-dimensional polyacrylamide gel electrophoresis (PAGE) as previously described.[14]

Immunological studies. Platelets were typed for the Pl^{A1} antigen by microcomplement fixation.[15] Platelet-associated IgG (PAIgG) was measured by radioimmunoassay.[16] Acid eluates were prepared from washed platelets of the patient (eluate W) and, as a control, from two healthy donors. Eluates were screened for immunoglobulin classes and subclasses by a modified enzyme-linked immunosorbent assay (ELISA) (courtesy of Dr Skvaril, Berne, Switzerland) and for platelet-binding antibodies by indirect immunofluorescence[17] applying a platelet panel from selected blood group O donors with known Pl$^{A1/A2}$, Bak^a, and HLA antigens, and a patient with GT. Immunoprecipitation[18] was carried out using eluate W and solubilized surface-labeled normal or GT platelets. The immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by fluorography.

The specificity of platelet autoantibodies in eluate W was further assessed by a solid-phase radioimmunoassay (SPRIA) as recently described by Woods et al.[7] Microtiter wells coated with mab specific for GPIb (FMC 25)[19] or GPIIb/IIIa (Gi5),[18] respectively, were reacted with either normal platelet extracts or, in control wells, extracts from monolayer cultures of a HeLa cell line; they were washed and then incubated with eluates, Pl^{A1} antibodies (N = 4), or quinine/quinidine-dependent antibodies (N = 3). After three additional washings, the amount of fixed antibodies was determined by a radioiodinated, affinity-purified F(ab')$_2$ goat anti-human IgG (Dianova, Hamburg, FRG). Values were expressed as the ratio of radioactivity bound to platelet extracts *v* HeLa extracts.

The effect of eluate W on platelet function was studied by two methods—first, by inhibition of platelet aggregation induced by ADP (final concentration 2 μmol/L) and, in some experiments, by collagen and ristocetin (final concentrations 5 μg/mL and 1.7 mg/mL, respectively). Control substances consisted of saline, pooled PPP, PPP of patient W, and normal platelet eluates. Second, the effect of eluate W was studied by inhibition of ^{125}I-fibrinogen binding. Platelets were washed by a modification of Mustard's method and adjusted to a concentration of $1.25 \times 10^5/\mu L$ with a

Tyrode's/albumin (TA) buffer containing 1 mmol of Ca^{2+}. Binding of ^{125}I-fibrinogen to platelets was measured as recently described.[20] In brief, 800 μL of washed platelets were incubated with 50 μL of eluate W or controls (TA buffer or normal eluate) for 20 minutes at 37 °C without stirring. Then, 100 μL of "hot" fibrinogen (a mixture of ^{125}I-labeled human fibrinogen (Amersham) and unlabeled fibrinogen (grade I, Kabi, Sweden) in a ratio of 1:6.25 (final fibrinogen concentration 0.1 mg/mL) and 50 μL of the aggregating agent were added and the mixture was stirred. At times indicated, two samples of 100 μL of the reaction mixture were centrifuged on a 20% sucrose gradient for two minutes at 10,000 g. The radioactivity bound to the platelet pellet was determined and used to calculate the amount of fibrinogen bound per 10^8 platelets.

RESULTS

Coagulation Data

All routine coagulation tests of plasma W were normal. Plasma levels for PF4 (2.9 ng/mL) and βTG (48.6 ng/mL) were within normal range [median values and 90% range of values of normal individuals (N = 40) were 6.1 ng/mL for PF4 (range 2.0 to 9.6 ng/mL) and 38.2 ng/mL for βTG (range 16.6 to 56.0 ng/mL)].

Platelet Data

Morphology. The platelets of the patient were slightly larger than normal (mean vol 12.4 fL), but did not show any other morphologic abnormalities by light or electron microscopy.

Function. The results of platelet function tests at various times during the entire observation period are summarized in Table 1. The data clearly reflect the fluctuating course of the disease. A profound defect of platelet function was demonstrable on first examination of the patient in 1978; it tended to normalize until 1982, relapsed in 1983 through 1984, and disappeared again in 1985. The defect was characterized mainly by prolonged bleeding time, decreased platelet retention and clot retraction, and failure of platelets to aggregate in response to collagen, ADP, arachidonic acid, epinephrine, and thrombin (data not shown), whereas ristocetin-induced agglutination was always normal. Platelet counts between 1978 and 1985 gave normal results. Any influence of the patient's plasma on aggregation was excluded by cross-over experiments. Washed platelets of the patient and a normal person were incubated with the patient's or normal PPP and their aggregability by ADP measured. Although the patient's platelets suspended in normal PPP did not respond at all, aggregation of normal platelets was unchanged, irrespective of the origin of PPP. ADP and ATP concentrations of the patient's platelets were also normal (3.43 and 4.81 μmol/L per 10^{11} platelets, respectively; normal values (x ± 1 SD; N = 8) were 4.37 ± 1.11 and 5.65 ± 0.86 μmol/L per 10^{11} platelets, respectively).

Membrane glycoprotein composition. Fluorograms of two-dimensional gel electrophoretic separations of surface-labeled platelets from the patient, a normal donor, and a patient with inherited GT are shown in Fig 1. Unlike those of GT, the patient's platelets clearly had normal amounts of GPIIb$_\alpha$, IIb$_\beta$, and IIIa in the normal positions. However, the gels from the patient showed additional protein bands at 200, 55, and 33 kd, probably corresponding to undissociated GPIIb/IIIa complex and to heavy and light chains of IgG.

Binding to platelets of mabs AN 51, J 15, and C 17 was tested in 1982 and twice in 1984. The three mabs always bound normally (ie, with the same fluorescence intensity as that of normal controls) to the patient's platelets. In contrast, the platelets of eight patients with GT did not react with mabs J 15 and C 17, whereas mab AN 51 always bound normally (data not shown).

Immunological studies. The platelets of the patient reacted normally with Pl^{A1} antibodies with a slightly increased background. PAIgG, expressed as percentage of total radioactivity, was greatly elevated (25%) during bad clinical condition but became normal (2.7%) when the patient recovered [median values were for normal individuals (N = 120) 2.3% (range 0.9% to 3.1%), for seven patients with GT 3.2% (range 2.4% to 5.6%)].

An eluate prepared from the patient's platelets (eluate W) contained IgG immunoglobulins of subclass IgG1 and showed strong platelet reactivity in indirect immunofluorescence with five of five panel platelets without Pl$^{A1/A2}$ or Bak^{a} specificity. It did not react with platelets from a patient with GT.

In immunoprecipitation experiments using eluate W as antibody and *Staphylococcus aureus* Cowan I as precipitant, eluate W specifically precipitated bands corresponding to GPIIb (both subunits), GPIIIa, and a band of 200 kd with extracts from normal, but not from GT platelets (Fig 2). The 55-kd and 33-kd species from solubilized labeled patient's platelets (before reduction) bound to Protein A-Sepharose (data not shown), providing additional evidence that they were heavy and light chains of IgG.

The effect of eluate W on platelet function was tested on ADP-induced platelet aggregation and fibrinogen binding. Inhibition of ADP-induced aggregation of normal PRP by a 1:10 dilution of eluate was already complete after five-minute preincubation time and did not change up to 60 minutes. Aggregation inhibition was dose dependent (Table 2 represents one of two experiments with similar results). Collagen-induced aggregation was also inhibited, but ristocetin-induced agglutination was unaffected (data not shown). None of the control substances, two normal eluates included (only one is given in Table 2), inhibited aggregation.

Inhibition of ADP-induced fibrinogen binding by eluate W is shown in Fig 3. Although ~1,500 ng fibrinogen per 10^8 platelets were bound in the presence of a control eluate (or buffer), there was almost no fibrinogen binding with eluate W.

The antibodies in eluate W were shown by radioimmunoassay to be against epitopes on GPIIb/IIIa (Table 3). Eluate W was strongly reactive with GPIIb/IIIa, but not with GPIb. Of the seven platelet-specific antibodies used for controls, four Pl^{A1} antibodies reacted similarly, whereas three quinine/quinidine-dependent antibodies showed the reverse pattern.

DISCUSSION

Patient W described in this report had a platelet function defect characteristic of GT. However, in contrast to the

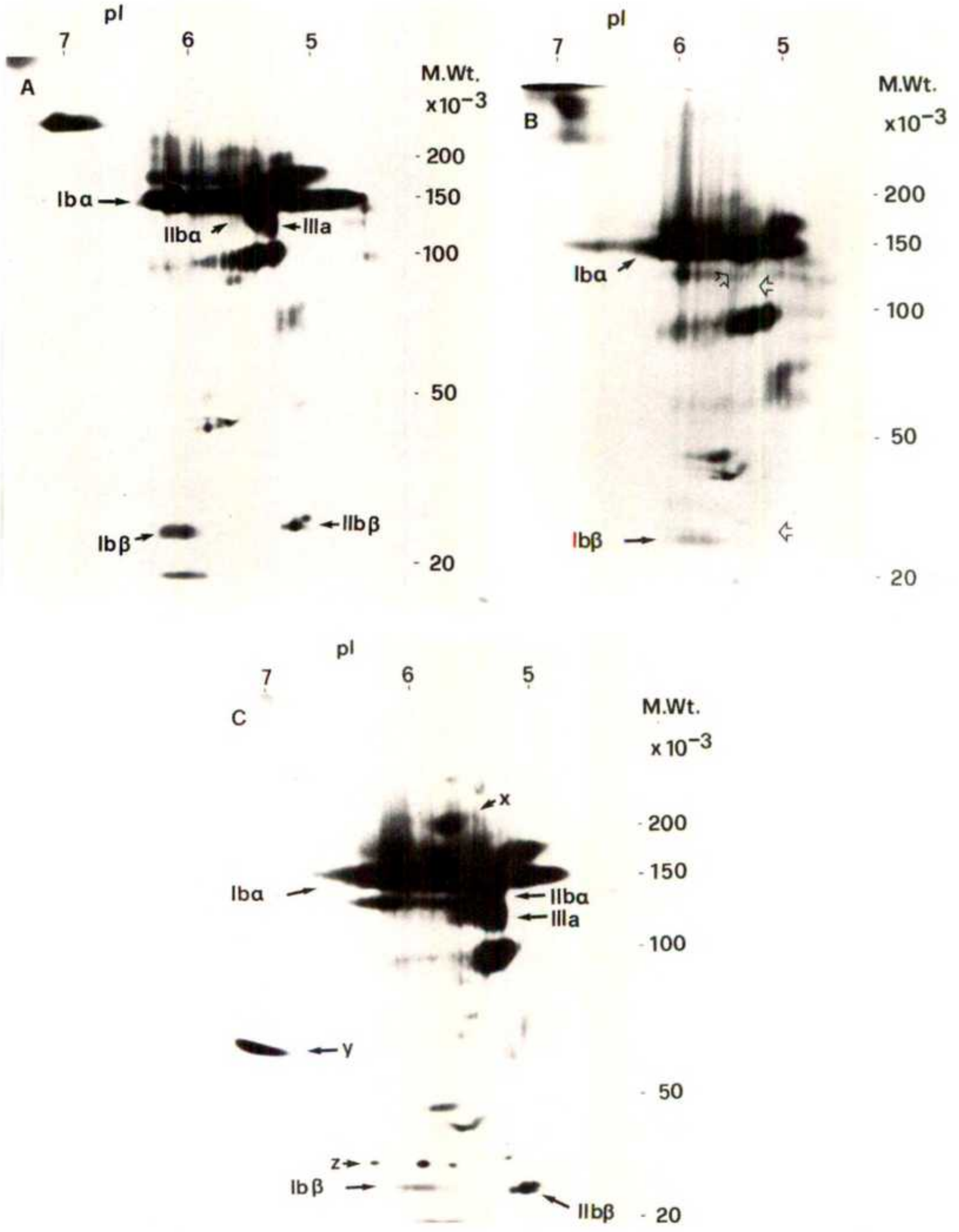

Fig 1. Fluorograms of two-dimensional polyacrylamide gels of platelets surface-labeled by the periodate/^{3}H-NaBH$_4$ method. (A) normal platelets. (B) Glanzmann's thrombasthenia platelets; the missing glycoproteins are indicated. (C) Platelets from patient W; labeled components not normally found are indicated as x, y, and z.

classic inherited form, her hemorrhagic diathesis, which developed during adult life, showed a remarkable fluctuation in both clinical symptoms and laboratory findings and, most important, GPIIb and IIIa were demonstrably normal by biochemical and immunological techniques. In gel electrophoretic separations, normal amounts of GPIIb (both subunits) and IIIa were seen in the normal positions, and the platelets reacted normally with P1^{A1} antibodies and mabs specific for GPIIIa and GPIIb/IIIa, respectively.

There is good evidence that this apparent discrepancy can be explained by an IgG1 antibody that could be demonstrated in an eluate prepared from the heavily IgG-loaded platelets of the patient. Immunological data clearly demonstrated specificity of the antibodies in eluate W against GPIIb/IIIa: (a) The eluate showed strong platelet reactivity with panel platelets without P1$^{A1/A2}$ or Bak^a specificity, but did not react with GT platelets, lacking GPIIb/IIIa; (b) eluate W was strongly reactive with normal platelet extracts enriched in the GPIIb/IIIa complex by adsorption with specific mab for the complex; (c) eluate W immunoprecipi-

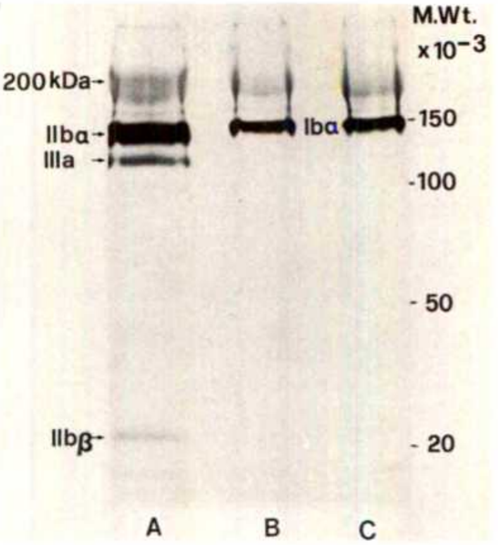

Fig 2. Fluorogram of a sodium dodecyl sulfate-polyacrylamide gel separation of immunoprecipitates from solubilized platelets, surface-labeled with the periodate/^{3}H-NaBH$_4$ method. (A) Normal platelets, immunoprecipitated with eluate from the patient's platelets. (B) Glanzmann's thrombasthenia platelets immunoprecipitated with eluate W. (C) Normal platelets immunoprecipitated with eluate from (different) normal platelets.

tated GPIIb (both units) and IIIa from solubilized normal platelets, but did not react with GT platelets. We speculate that the additional 200-kd band precipitated from normal extracts and the higher mol wt spot not seen normally in gel electrophoretic separations of platelet membranes (marked "x" in Fig 1C) corresponds to undissociated GPIIb/IIIa.

The specificity of the antibodies in eluate W against GPIIb/IIIa is further supported by their effect on platelet functions closely related to the receptor GPIIb/IIIa. Eluate W strongly inhibited ADP-induced and collagen-induced aggregation and also ADP-induced fibrinogen binding in normal PRP.

From the patient's medical history, it is highly probable that her hemorrhagic diathesis developed during adult life. Together with the fluctuating course and evidence of an antibody, this precludes a variant of inborn GT characterized by structural abnormalities of GPIIb/IIIa.[21,22] Furthermore, the patient had no history of transfusion before onset of the hemorrhagic diathesis or of other events known to cause possible alloimmunization. Therefore, an alloantibody, as reported in a patient with inherited thrombasthenia who received multiple transfusions,[23] can also be excluded. Considering all the data, it is very likely that in patient W the GPIIb/IIIa-reactive antibody was due to an autoimmune reaction.

Platelet functional defects resulting from antiplatelet antibodies have been reported in autoimmune disorders such as ATP in remission, systemic lupus erythematosus, and in the

Table 2. Inhibition of ADP-Induced Aggregation in Normal PRP by Eluate W

Incubation Mixture	ADP (2.0 μmol/L*)-Induced Platelet Aggregation (max ΔOD/t, mm)
Normal PRP	348
Normal PRP + saline	384
Normal PRP + normal PPP	385
Normal PRP + W's PPP	253
Normal PRP + normal eluate	282
Normal PRP + eluate W (1:10*)	0
Normal PRP + eluate W (1:20*)	57
Normal PRP + eluate W (1:40*)	205
Normal PRP + eluate W (1:80*)	305
Normal PRP + eluate W (1:160*)	303
Normal PRP + eluate W (1:320*)	326
Normal PRP + eluate W (1:640*)	372

Nine volumes of normal PRP were mixed with 1 vol of eluate at various dilutions or control substances, respectively.

*Final concentrations.

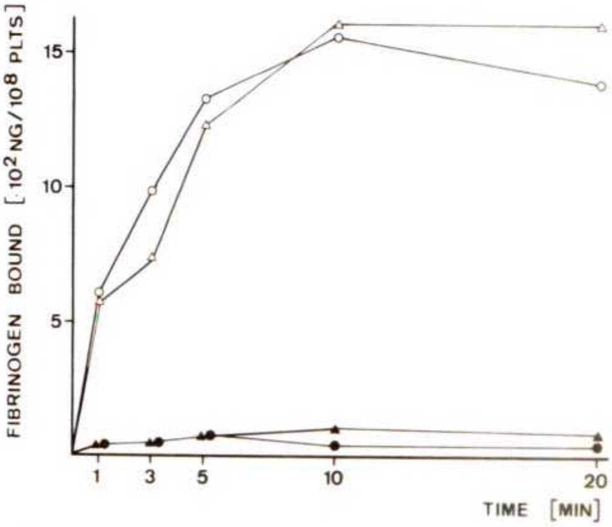

Fig 3. Inhibition of ADP-induced fibrinogen binding to washed platelets by eluate W. Washed platelets were preincubated with eluate W (solid triangles), control eluate (open triangles), or buffer (open circles), respectively, without stirring. A mixture of ^{125}I-labeled and unlabeled fibrinogen (final concentration 0.1 mg/mL) and ADP were then added simultaneously, the reaction was stirred, and samples taken at times indicated were investigated for platelet-bound reactivity (details given in Materials and Methods section). Solid circle, nonspecific binding of ^{125}I-fibrinogen in the absence of ADP (N = 7).

Table 3. Demonstration of Specificity of Antibodies in Eluate W to Platelet Glycoproteins IIb/IIIa, as Compared to P1^{A1} Antibodies and Quinine/Quinidine-dependent Antibodies

Antibodies	Glycoproteins Bound by Mab Specific for GPIIb/IIIa	Glycoproteins Bound by Mab Specific for GPIb
Normal sera (N = 29)	0.89 ± 0.09	0.90 ± 0.09
Pl^{A1} antibodies (N = 4)	7.17 ± 0.99	1.02 ± 0.03
Quinine/Quinidine-dependent antibodies (N = 3)	0.83 ± 0.01	2.87 ± 1.46
Eluate W	46.20	1.10
Control eluate	1.10	0.80

Data are expressed as a ratio ($\bar{x} \pm s$) of radioactivity bound to the normal platelet extract compared to radioactivity bound to the HeLa control extract.

easy-bruising syndrome.[24] However, the lesion in all these cases, with the characteristics of an aspirinlike defect or storage pool disease, was fundamentally different from the findings in this report. Recently, autoantibodies directed against GPIIb/IIIa were reported in some patients with ATP[5-7]; platelet function investigations were not performed in these studies. However, it must be stressed that in patient W, with platelet counts in the normal range during the entire observation period, the most important diagnostic criterion of ATP is not fulfilled. The increased number of megathrombocytes supports the assumption of subclinical compensated autoimmune thrombocytolysis. However, taking into account the high level of platelet-bound IgG in patient W on the one hand, and the correlation of platelet-bound IgG with the degree of thrombocytopenia on the other hand,[24] this is unlikely. It is conceivable that the antibody in patient W is directed against an epitope of GPIIb/IIIa that is critically involved in platelet function but has no significant relation to thrombocytolysis, in contrast to binding sites of autoantibodies in chronic ATP.

Considering the clinical course and the biochemical and immunological data, it is very likely that the acquired GT-like hemorrhagic diathesis in this patient is caused by an autoantibody directed against GPIIb/IIIa. Other reports on acquired platelet dysfunctions resembling congenital functional platelet defects may be expected, as indicated by a recent report on an acquired Bernard-Soulier syndrome.[25]

ACKNOWLEDGMENT

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Chronic T Cell Leukemia With Unusual Cellular Characteristics in Ataxia Telangiectasia

By Ulrich Dührsen, Michael Uppenkamp, Ingeborg Uppenkamp, Reinhard Becher, Marianne Engelhard, Erika König, Peter Meusers, Stefan Meuer, and Günter Brittinger

A 27-year-old male patient with ataxia telangiectasia (AT) developed atypical chronic lymphocytic leukemia with increasing bone marrow infiltration in the absence of organomegaly. One-third of the leukemia cells expressed a mature suppressor/cytotoxic T cell phenotype ($T3^+$ $T4^-$ $T6^-$ $T8^+$ $T10^-$), two-thirds demonstrated additional helper/inducer T cell-associated antigens ($T3^+$ $T4^+$ $T6^-$ $T8^+$ $T10^-$), and a small fraction reacted with a natural killer (NK) cell-specific monoclonal antibody (Leu 11^+). The proliferative response to stimulation in vitro with lektines and various monoclonal antibodies resembled the proliferation pattern of mature thymocytes: The cells responded to phytohemagglutinin (PHA), concanavalin A (ConA), stimulation of the T3-Ti receptor complex with Sepharose-bound anti-T3, and stimulation of the sheep erythrocyte receptor protein with anti-$T11_2$ and anti-$T11_3$ in conjunction with exogenous interleukin-2 (IL 2); they failed, however, to proliferate after stimulation with anti-$T11_2$ and anti-$T11_3$ alone. There was no response in the mixed lymphocyte reaction (MLR) and no suppression of the MLR between two healthy donors. Antibody-dependent cell-mediated cytotoxicity and NK activity could not be demonstrated. Cytogenetic analysis revealed complex clonal aberrations, including an interstitial deletion of the long arm of chromosome 14 concerning bands q21-31, loss of chromosome 20, and loss of the Y chromosome. Cytostatic chemotherapy was of little use and caused serious side effects, whereas leukapheresis proved effective in reducing the tumor load. The clinical data and laboratory findings in this case correspond to three previously described patients with AT who developed chronic T cell leukemia. Thus, in adult patients with AT, malignant proliferation of cytogenetically marked and phenotypically heterogeneous mature T cells seems to be a frequent complication.

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ATAXIA telangiectasia (AT) is an autosomal recessive syndrome characterized by progressive cerebellar ataxia, oculocutaneous telangiectases, and a complex primary immunodeficiency predisposing to frequent and severe respiratory infections; in addition, various endocrine, hepatic, and integumental abnormalities may be associated.[1] Patients with AT have an elevated risk of developing malignancies (~10%), especially malignant lymphoma and leukemia.[2] Because death usually occurs during childhood from respiratory failure or neoplastic diseases, few patients reach the third or fourth decade of life. Among these patients, development of chronic T cell leukemia (T-CLL) has repeatedly been observed.[3-8]

In this report, we describe an adult male patient with AT complicated by T-CLL. The clinical data and laboratory findings in our patient reveal striking similarities with the previously reported cases. Thus, T-CLL in adults with AT may constitute a well-defined subentity in the heterogeneous group of the chronic T cell neoplasms.

MATERIALS AND METHODS

Case Report

K-D.B. is a male, born in 1955. From his second year of life, the patient developed a slowly progressive incoordination of gait, hand movements, and speech, which confined him to a wheel chair at the age of 6 years. When he was 20 years old, his parents first noticed ocular and cutaneous telangiectases, predominantly in the antecubital areas, on the back of the hands and on the auricles. Apart from pneumonia following rubeola in 1959 and herpes zoster of the left trigeminal nerve in 1981, there was no history of severe infections. A liver biopsy performed in 1983 because of elevated serum transaminase activities revealed diffuse fatty infiltration with minimal chronic inflammation.

In July 1983, the patient was first admitted to our hospital for evaluation of leucocytosis. He was of small stature (165 cm) and slightly underweight (55 kg). Physical examination showed several vitiligolike skin areas and mild bilateral gynecomastia in addition to oculocutaneous telangiectases. The right testicle was not palpable; the size of the left one was in the lower normal range. He had no enlarged lymph nodes and no splenomegaly. Neurological examination revealed severe cerebellar ataxia with ocular dysmetria, nystagmus, dysarthric speech, inability to stand or walk without help, intentional tremor, and dysdiadochokinesis. The tendon reflexes were absent; vibration sense was diminished in a socklike fashion.

Clinical diagnosis of AT was made and was corroborated by reduced serum concentrations of IgA (<42 mg/dL) and IgE (<5 U/mL), contrasting with increased concentrations of IgM (702 mg/dL), α-1-fetoprotein (359 ng/mL) and carcinoembryonic antigen (3.0 ng/mL), and IgG levels in the upper normal range (1,762 mg/dL). The oral glucose tolerance test reflected impaired carbohydrate tolerance[9] due to insulin resistance (fasting venous blood concentrations: glucose 70 mg/dL, insulin 55 μU/mL; two-hour values: glucose 175 mg/dL, insulin >200 μU/mL). The result of the LH-RH test was compatible with primary hypogonadism (basal serum concentrations: LH 13.3 mU/mL, FSH 19.4 mU/mL; maximum values after stimulation: LH 45.3 mU/mL, FSH 28.9 mU/mL).

Hemoglobin concentration was 13.7 g/dL, leucocyte count was 52,500/μL with 92% atypical lymphocytes, and platelet count was 436,000/μL. Bone marrow aspirate was consistent with moderate patchy infiltration with medium-sized lymphocytes. Chest x-ray films, sonography of the abdomen, and total body computed tomography (CT) revealed neither lymphadenopathy nor other organomegaly. Further analysis of the atypical cells indicated chronic T cell leukemia. Antibodies against HTLV I could not be demonstrated by various techniques [enzyme-linked immunosorbent assay (ELISA),

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radioimmunoprecipitation]. Because symptoms were lacking, a specific therapy was not initiated.

In March 1984, continuous increase of the leucocyte count up to 230,000/μL prompted us to start antineoplastic chemotherapy. Because the biweekly administration of chlorambucil and prednisone proved ineffective in preventing a further rise of leucocytes up to 300,000/μL, chemotherapy was intensified by continuous treatment with cyclophosphamide (100 to 150 mg daily) and prednisone (25 to 50 mg daily). After 4 weeks of therapy, the patient's leucocyte count dropped to 170,000/μL, but a severe hemorrhagic cystitis with incomplete recovery forced us to discontinue this treatment. In November 1984, the leucocytosis exceeded 800,000/μL, and small pleural effusions developed; the patient complained of dyspnea, dizziness, and general fatigue. Bone marrow was now densely infiltrated, but apart from slight thrombocytopenia, the patient still had no important hematopoietic insufficiency, organomegaly, or significant change in serum immunoglobulin concentrations. Intermittent leukapheresis performed at intervals of 15 to 30 days led to a steady reduction of the WBC count to 400,000 to 500,000/μL, followed by a temporary improvement of the patient's general condition. From March 1985 on, the cyclophosphamide-induced cystitis repeatedly worsened, and the patient was debilitated by nearly continuous urinary bleeding. He died on June 2, 1985, of severe hemorrhage from the bladder. Permission for autopsy was denied.

In accordance with institutional guidelines the patient was advised of all diagnostic and therapeutic procedures, including attendant risks, and gave informed consent.

Phenotypic Characterization

Cytomorphology and cytochemistry. Peripheral blood and bone marrow smears were stained with a modified May-Grünwald-Giemsa stain. Periodic-acid Schiff (PAS) and acid phosphatase reactions were performed using routine techniques.

Terminal deoxynucleotidyl transferase (TdT). TdT was determined by indirect immunofluorescence on fixed mononuclear cells with specific rabbit anti-TdT antiserum and fluorescein-conjugated goat anti-rabbit antibodies (BRL Terminal Transferase Immunofluorescent Assay Kit, Gaithersburg, Md).

Surface marker analysis. Mononuclear cells from heparinized peripheral blood (PBMCs) and bone marrow samples (BMMCs) were separated by Ficoll sodium metrizoate gradient centrifugation (Lymphoprep, Nyegaard, Oslo) using standard techniques.[10,11]

Surface antigens defined by monoclonal antibodies were analyzed by indirect immunofluorescence. After incubation of 1×10^6 cells with monoclonal mouse antibodies, cells were washed twice and then reincubated with an affinity-purified fluorescein-conjugated rabbit anti-mouse immunoglobulin as a second layer (Dakopatts, Copenhagen). The monoclonal antibodies used were commercially available products (Ortho Diagnostic Systems, Raritan, NJ, Becton Dickinson Laboratory Systems, Moutain View, Calif); Coulter Immunology, Hialeah, Fla) that have been characterized in detail.[12-16] In brief, pan-T antigens were detected by OKT 3, OKT 11 (E receptor), anti-Leu 1, and anti-Leu 4; inducer/helper T cell-associated antigens were detected by OKT 4 and anti-Leu 3a; suppressor/cytotoxic T cell-associated antigens were detected by OKT 8 and anti-Leu 2a; thymic antigens were detected by OKT 6, OKT 9 (transferrin receptor), and OKT 10; the HLA-DR antigen complex was detected by OKI a-1; the cALL antigen was detected by C-J 5; monocyte and macrophage associated antigens were detected by OKM 1 and anti-Leu M 3; natural killer (NK) cell-associated antigens were detected by anti-Leu 11; and B cell-specific antigen was detected by C-B 1. These antibody studies were done on fresh cells; only the anti-Leu 8 monoclonal antibody, which was tested for further dissection of the helper/inducer and suppressor/cytotoxic subsets,[16] was applied to cryopreserved and thawed mononuclear cells.

Direct two-color immunofluorescence with fluorescein- and phycoerythrin-conjugated monoclonal antibodies was used in determining the percentage of cells simultaneously expressing Leu 2a and Leu 3a markers.

Positive cells were enumerated by a fluorescence activated cell sorter (FACS II, Becton Dickinson) or by fluorescence microscopy (Orthoplan, Leitz, Wetzlar, FRG). By both techniques[17] identical patterns of surface antigen expression were found (data not shown).

Spontaneous rosette formation with sheep erythrocytes (E rosettes) was assessed by adding a suspension of lymphoid cells to sheep cells in the presence of absorbed and inactivated fetal calf serum (FCS) at 37 °C. After cells were centrifuged and incubated overnight at 4 °C, the percentage of E rosette-forming cells was determined microscopically.

Functional Studies

Proliferative assays. Mitogen responsiveness was assayed by incubating 1.5×10^5 PBMC with 4.21 μg of phytohemagglutinin (PHA-P, Difco, Detroit), 35.57 μg of concanavalin A (Con A, Difco), or 3.48 μg of pokeweed-mitogen (PWM; GIBCO, Grand Island, NY), respectively. The cells were cultured in RPMI 1629 with glutamine (GIBCO) and 13% inactivated FCS (final volume, 240 μL) at 37 °C for 68 hours. Two microcuries of ^{3}H-methylthymidine were added for an additional four hours. The cells were then harvested on paper strips, and ^{3}H-methylthymidine incorporation was determined by scintillation counting. A stimulation index (SI) was calculated as the ratio of cpm in stimulated and control cultures.[18]

To further characterize the proliferative behavior of the patient's cells, a second set of experiments was performed. Proliferation was determined in response to PHA-P (Wellcome, Burgwedel, FRG), to Sepharose-bound anti-T3 monoclonal antibody,[19,20] to stimulation of the E rosette receptor protein with anti-T11$_2$ and anti-T11$_3$ monoclonal antibodies[21] and to stimulation with anti-T11$_{2/3}$ plus exogenous interleukin 2 (IL 2).[22] All experiments were done both with the unseparated leukemia cells and after separation of the cells into the two main tumor cell subsets (T3$^+$ T4$^+$ T8$^+$ and T3$^+$T4$^-$ T8$^+$). PBMC were obtained as described above. Separation of the T4$^+$ and T4$^-$ subsets was achieved either by indirect immunofluorescence on an EPICS C cell sorter (Coulter) or by selectively lysing the T4$^+$ subset. For the latter purpose, the unseparated PBMCs were incubated with anti-T4 for one hour at 4 °C. Subsequently, rabbit complement was added, and the suspension was incubated for one hour at 37 °C in a shaking water bath; the cells were then washed repeatedly.

Recombinant IL 2 was a generous gift of Sandoz Research Institute (Vienna). Anti-T11$_2$ and anti-T11$_3$ monoclonal antibodies were kindly provided by Dr E.L. Reinherz (DFCI, Boston), and anti-T3, anti-T4, and anti-T8 were purchased from Coulter (Krefeld, FRG). For surface coupling of anti-T3, the monoclonal antibody was purified with Sepharose Protein A (Pharmacia, Uppsala, Sweden) and then covalently linked to CNBr-activated Sepharose 4 B (Pharmacia) at a concentration of 3 mg of purified antibody per milliliter of swollen Sepharose beads.[19]

Responder cells (3×10^4) were incubated with various stimuli in round-bottomed microtiter wells (Costar, Cambridge, Mass) in 200 μL of final culture medium RPMI 1640, supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 2% glutamine (GIBCO, Paisley, Scotland). PHA was added at a concentration of 0.25 μg/mL, and anti-T3 carrying Sepharose beads were suspended at dilutions previously determined to yield optimal proliferative responses. Stimulation with PHA and anti-T3 Sepharose was performed in the presence or absence of 5% adherent cells (6,000 rad irradiated) (Mϕ) of a healthy donor. Anti-T11$_2$ and anti-T11$_3$ antibodies were used together at a final concentration of 1:300, with or without addition of recombinant IL 2 at a concentration of 5 ng/mL.

After three days of in vitro culture in a 7% CO$_2$ humidified atmosphere at 37 °C, wells were pulsed with 1 μCi of ^{3}H-thymidine for 16 hours and mashed with a Titertek cell harvester (Flow, Meckenheim, FRG). ^{3}H-Thymidine uptake was measured in a Packard liquid scintillation spectrometer (Packard Instrument Co, Inc, Downers Grove, Ill). The results obtained for the patient's PBMCs were compared with those of purified T cells of a healthy donor. The method of T cell purification has been described previously and included Ficoll-Hypaque density centrifugation, removal of adherent cells on glass Petri dishes, separation of E rosette-positive cells, lysis of RBCs with ACK-buffer, and lysis of remaining monocytes by treatment with monocyte-specific monoclonal antibodies plus rabbit complement.[23] Results were expressed as cpm, all values representing the mean of triplicate experiments. SDs were ≤ 15%.

Mixed lymphocyte reaction (MLR). PBMCs obtained from the patient and two healthy donors as described above were used as stimulators and responders. In addition, response to stimulation with an irradiated Epstein-Barr Virus (EBV)-transformed human lymphoblastoid B cell line (B-LCL) was determined. After irradiation (6,000 rad) of stimulator cells, 5×10^4 stimulator and 5×10^4 responder cells were incubated in standard medium under the above described conditions for five days. After being pulsed with 1 μCi of ^{3}H-thymidine for 18 hours, cells were harvested, and ^{3}H-thymidine uptake was measured as above. Results were expressed as cpm (means of triplicate experiments). SDs were ≤15%.

Cytotoxic activity. Antibody-dependent cell-mediated cytotoxicity (ADCC) was measured v ^{51}Cr-labeled rabbit-antibody sensitized RL δ 1 lymphoblastoid cells. Natural killer (NK) activity was determined by adding PBMCs to ^{51}Cr-labeled K 562 target cells in the presence or absence of human sera containing NK-stimulating factors. Specific cytotoxicity was calculated as the ratio of cpm in the supernatant (after subtraction of controls) and total cpm in the target cells, multiplied by 100%.[24]

Cytogenetic Studies

Cultures of PHA-stimulated lymphocytes were set up in RPMI 1640 medium containing 20% FCS, and were harvested after 72 hours, which included a treatment with colcemide (0.02 μg/mL) for the last two hours. Thereafter, cells were treated with hypotonic KCl (0.075 mol/L) and fixed in methyl alcohol–acetic acid (3:1). G-banding was performed according to conventional methods after short-term treatment with trypsin.[25] Karyotypic analysis was done according to the guidelines of the Paris Conference.[26]

RESULTS

Phenotypic Characterization of Leukemic Cells

The peripheral blood smear showed a predominance of medium-sized, pleomorphic lymphocytes with irregularly shaped, partly indented nuclei and moderate cytoplasm (Fig 1). Cytochemically, most cells displayed a dotlike activity of acid phosphatase; the PAS reaction and immunofluorescent staining of TdT were negative.

Eighty-nine percent of the PBMCs formed rosettes with sheep erythrocytes. From July 1983 to August 1984, the surface antigens of the PBMCs were analyzed on seven occasions. Despite an increase in the absolute number of leukocytes from 52,500 to 406,000/μL, all examinations yielded comparable results (Table 1, Fig 2). Approximately 98% of the PBMCs reacted strongly with pan-T and suppressor/cytotoxic T cell reagents. Approximately 60% of the cells showed a (weaker) reaction with anti-helper/inducer T cell antibodies. Double staining with anti-Leu 3a and anti-Leu 2a

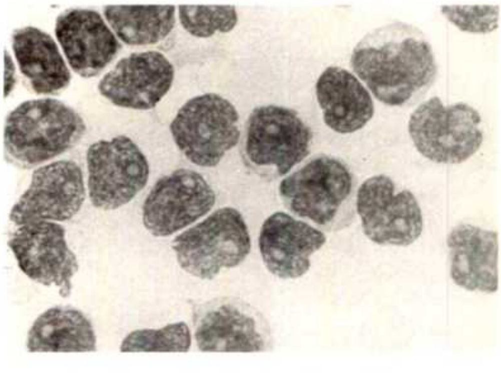

Fig 1. Peripheral blood smear.

Table 1. Surface Marker Profile of PBMC and BMMC

	Positive Cells (%)	
Cells	PBMCs	BMMCs
Pan-T cell antigens		
T3	97 (79–98)	46
T11	98 (94–99)	40
Leu 1	97 (94–98)	ND
Leu 4	98 (96–99)	ND
Helper/inducer T cell subset		
T4	56 (48–64)	6
Leu 3a	61 (56–69)	ND
Suppressor/cytotoxic T cell subset		
T8	98 (93–98)	40
Leu 2a	98 (96–99)	ND
Thymocytes		
T6	2 (1–3)	4
T9	0	6
T10	0	1
Subdivisions of T4$^+$ and T8$^+$ cells		
Leu 8	1 (0–3)	ND
NK cells		
Leu 11	7 (3–9)	ND
HLA-DR complex		
Ia-1	4 (1–5)	6
B lymphocytes		
B1	2 (1–3)	ND
Monocytes/macrophages		
M1	2	53
Leu M3	1 (1–2)	ND
cALL antigen		
J5	0	5

Peripheral blood mononuclear cell (PBMC) profile is median and range of seven analyses; bone marrow mononuclear cell (BMMC) profile was determined once. ND, not determined.

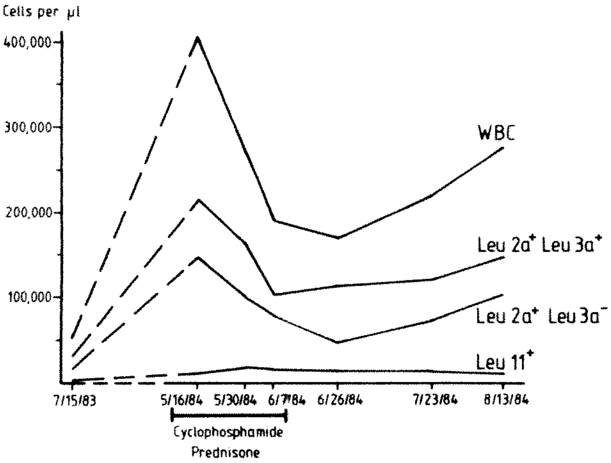

Fig 2. Variation of the absolute count of total leukocytes (WBC) and cells with different phenotypes from July 1983 to August 1984. The proportion of cells reacting with anti-Leu 2a and anti-Leu 3a, respectively, was determined by single marker analysis. Because double staining showed that all Leu 3a$^+$ cells coexpressed Leu 2a and that a smaller proportion of cells exhibited positivity only for Leu 2a, the Leu 2a$^+$ Leu 3a$^-$ subset was determined by subtracting the number of Leu 3a$^+$ cells from the total number of cells reacting with anti-Leu 2a. Bar indicates period of cytotoxic therapy.

was performed on four occasions; 95% (range 93% to 95%) of the PBMCs were positive for Leu 2a, and 55% were positive (52% to 58%) for Leu 3a. All cells staining for Leu 3a simultaneously reacted with anti-Leu 2a; about one-third of the cells, however, exhibited only positivity for Leu 2a. The fraction of cells that reacted with anti-Leu 11 was in the normal range; with regard to the increased absolute number of PBMCs, however, the number of Leu 11$^+$ cells was distinctly raised whenever examined (Fig 2). The panel of monoclonal antibodies tested failed to demonstrate an increased expression of antigens associated with thymocytes, B lymphocytes, and monocytes.

The surface marker profile of the BMMCs was analyzed only once. Due to a high proportion of monocytes (OKM 1$^+$ cells: 53%) the number of cells staining for anti-T cell antibodies was correspondingly lower. The relation of the BMMCs carrying pan-T cell, suppressor/cytotoxic T cell, and thymocyte-associated antigens was comparable to the PBMCs, whereas the proportion of cells expressing helper/inducer T cell-associated antigens was lower.

Functional Characterization of Leukemic Cells

At the time of investigation, the absolute count of PBMCs varied between 300,000 and 400,000/µL with 98% T 8$^+$ cells; admixture of normal cells was therefore assumed to be very small.

Proliferative response to various stimuli. Proliferation after stimulation with ConA was normal (SI 11.6; normal range 7 to 90). Response to PHA was slightly reduced (SI 13.9; normal range 14 to 112). Response to PWM was markedly reduced (SI 0.8; normal range 2 to 80).

The proliferative behavior was further investigated in a second set of experiments (Table 2). The response to PHA of both the patient's cells and purified T cells derived from a healthy donor was grossly enhanced after addition of Mø to the culture medium. Stimulation with Sepharose-bound anti-T3 through the T3-Ti receptor complex[19,20] also required the presence of macrophages, under these conditions resulting in a high proliferative response of both the patient's and the healthy donor's cells. Although the patient's leukemia cells showed virtually the same response to PHA and Sepharose-bound anti-T3 as did the healthy donor's purified T cells, triggering of the E rosette receptor protein with anti-T11$_2$ and anti-T11$_3$[21] induced proliferation only of the healthy donor's cells, not the patient's cells. However, if exogenous IL-2 was added to the patient's anti-T11$_{2/3}$-treated cells, substantial proliferation was observed. In contrast, proliferation of the healthy donor's anti-T11$_{2/3}$-treated cells could not be further enhanced by addition of IL 2. IL 2 alone proved to be a very weak stimulus for the donor's purified T cells and was not capable of inducing proliferation of the patient's cells. The proliferation pattern was identical in the patient's unseparated cells and in the T3$^+$T4$^+$T8$^+$ and T3$^+$T4$^-$T8$^+$subsets, respectively.

Mixed lymphocyte reaction. In the first set of experiments, the MLR between the patient and two healthy donors was studied. As shown in Table 3, the patient's PBMCs

Table 2. Proliferative Response of the Patient's Unseparated and Separated PBMCs (T4$^+$ and T4$^-$ Subsets) to Various Stimuli in Comparison to Response of Purified T Cells of Healthy Donor

	Patient				Healthy Donor
		Cell Sorter-Separated Cells		Anti-T4 + Complement-Treated Cells	
Stimulus	Unseparated Cells	T3$^+$T4$^+$T8$^+$	T3$^+$T4$^-$T8$^+$	T3$^+$T4$^-$T8$^+$	Purified T Cells
Medium	204	195	113	86	181
IL 2	174	142	97	147	580
PHA	334	ND	ND	224	1,374
PHA + MØ	23,023	19,272	27,663	23,310	33,550
Anti-T3-Sepharose	194	104	151	219	ND
Anti-T3-Sepharose + MØ	11,484	10,042	11,977	10,873	12,270
Anti-T11$_2$ + anti-T11$_3$	356	262	179	100	25,260
Anti-T11$_2$ + anti-T11$_3$ + IL 2	5,516	4,187	5,220	5,060	24,289

PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; IL 2, interleukin 2; Mø, irradiated macrophages; ND, not determined. Results are expressed as cpm ^{3}H-thymidine uptake; SD ≤ 15%.

Table 3. Response of Patient's PBMC (P) and PBMC of Two Healthy Donors (D_1/D_2) in MLR

	Responder Cell Origin				
Stimulator Cells	P	D_1	D_2	P + D_1	P + D_2
Medium	110	135	183	145	94
P ↯	—	183	265	—	—
D_1 ↯	145	—	9,271	—	9,596
D_2 ↯	94	9,561	—	8,528	—
B-LCL ↯	561	48,965	52,112	54,690	55,111

Results are expressed as cpm ^{3}H-thymidine uptake; SD ≦ 15%. B-LCL ↯ alone yielded 504 cpm. ↯ = irradiated (6,000 rad).

PBMC, peripheral blood mononuclear cells; MLR, mixed lymphocyte reaction; B-LCL, Epstein-Barr virus-transformed human lymphoblastoid B cell line.

neither responded to stimulation with the donors' PBMCs nor to stimulation with B-LCL. Similarly, the patient's irradiated cells could not stimulate the responder cells of either donor. The MLR between the two donors and each of them *v* B-LCL, however, yielded high stimulative responses.

To exclude a suppressive effect of the patient's cells on the MLR, a second set of experiments was performed in which 5 × 10^4 nonirradiated cells of the patient were added to the mixed lymphocyte culture set up between the two donors or between each of the donors and B-LCL. The patient's cells did not influence the stimulative response of either healthy donor (Table 3).

Cytotoxic activity. As compared with those of a healthy control the patient's ADCC and NK activities were extremely low. Specific cytotoxicity for ADCC was 2.5% (control 47.5%), for NK activity in the absence of stimulating sera 0.5% (control 25.0%), and 2.0% to 4.0% for NK activity in the presence of stimulating sera (control 33.0% to 45.5%).

Cytogenetic Characterization of Leukemic Cells

Chromosome analysis revealed an abnormal hypodiploid clone with <10% normal cells left. The karyotype was described as 44X,−Y,−20,4q−,6p−,14q−,19p+, 20q+,22q−; the structural anomalies were interpreted as rec t(4;20)(q13;q12), interstitial del14(q21;q31), t(6;19;22)(p11;p13;q13). Although the 14q1 region seemed untypically condensed in three karyotypes, the breakpoint at 14q was clearly below 14q11. The Y chromosome was lost only in abnormal cells.

DISCUSSION

Subacute or chronic leukemias of mature T cells constitute a heterogeneous group of diseases. Clinical and laboratory findings allow the definition of several subentities, such as Sézary's syndrome and HTLV I-associated adult T cell leukemia of Japan,[27,28] both phenotypic helper cell disorders.[29,30] The term "chronic lymphocytic leukemia of T cell origin" has been applied to a number of derangements with variable clinical features.[31,32] Some of these are now being subsumed under the term of Tγ-lymphoproliferative disease, a proliferation of cytotoxic lymphocytes[33] of monoclonal or polyclonal origin.[34,35]

Our patient's leukemia does not correspond to any of the previously defined categories. Phenotypically, at least two different T cell subsets could be defined: Although a minor proportion of cells exhibited the mature suppressor/cytotoxic T cell phenotype ($T3^+T4^-T8^+$, Leu 4^+ Leu $3a^-$ Leu $2a^+$), about two-thirds of the PBMCs carried both suppressor/cytotoxic and helper/inducer T cell associated antigens ($T3^+T4^+T8^+$, Leu 4^+ Leu $3a^+$ Leu $2a^+$). Further characterization was achieved by analyzing the proliferation pattern in response to various stimuli. T cell activation with Sepharose-bound anti-T3 mimics triggering of the T cell receptor by antigen[19,20] and is known to induce changes similar to those induced by stimulation with PHA.[23] Although resting T cells proliferate in response to PHA or stimulation of the T3–Ti receptor complex only if monocytes are present to provide an additional signal (possibly IL1), preactivated HLA-DR positive T cells may proliferate even in the absence of monocytes.[23] In keeping with the lack of expression of HLA-DR antigens, our patient's cells behaved like resting T cells, proliferation being observed only if monocytes were added to the culture medium.

Apart from stimulation of the T3–Ti receptor complex, T cells can also be activated by the T11 sheep erythrocyte receptor protein.[21] This mode of activation is antigen independent and requires simultaneous triggering of two distinct epitopes ($T11_2$, $T11_3$) of the sheep erythrocyte binding protein by means of anti-$T11_2$ and anti-$T11_3$ monoclonal antibodies.[21] The physiological role of this "alternative pathway" of T cell activation and the natural ligand of the T11 receptor are still unknown.[21] Although peripheral T cells proliferate in response to both the antigen-dependent mechanism and the alternative path of activation by an IL 2-dependent autocrine mechanism,[19,21] it has been shown that thymocytes, although expressing the T11 receptor in almost all maturational stages, fail to proliferate after stimulation with anti-$T11_2$ and anti-$T11_3$.[22] However, if exogenous IL 2 is added to the anti-$T11_{2/3}$-treated cells, substantial proliferation occurs, indicating that—in contrast to peripheral T lymphocytes—triggering of $T11_2$ and $T11_3$ epitopes of thymocytes results only in expression of IL 2 receptors, but not in IL 2 secretion.[22] Although the T10 antigen that is usually encountered in late stages of thymic maturation[12] was not present on the patient's leukemia cells, their stimulation pattern was characteristic of mature thymocytes, proliferation being induced by PHA, Sepharose-bound anti-T3, and anti-$T11_{2/3}$ in conjunction with exogenous IL 2, but not by anti-$T11_{2/3}$ alone. No differences were found between the $T3^+$ $T4^+$ $T8^+$ and the $T3^+$ $T4^-T8^+$subsets.

The phenotype of the largest tumor cell subset ($T3^+$ $T4^+T6^-$ $T8^+$ $T10^-$ $T11^+$) has repeatedly been observed in T cell-derived non-Hodgkin lymphomas[36,37] and leukemias.[38-41] It is apparently not linked to a homogeneous clinical picture, because the patients' survival times ranged from a few weeks[38] to many years.[39] None of the other patients with a T cell neoplasm of this phenotype suffered from AT, and no details were given as to additional tumor cell populations.

The patient's cells did not respond to allogeneic leukocytes in the mixed lymphocyte culture. Because the MLR between two normal donors was not influenced by the patient's PBMCs, the negative proliferative response could not be ascribed to a suppressive effect of the tumor cells. Moreover, it is unlikely that the general immunodeficiency associated with AT was responsible for the negative MLR because in contrast to other T cell-dependent functions, the MLR is normal in most patients with AT.[42] Because chromosome analysis showed that >90% of the PHA-stimulated blood cells had the same pathological karyotype and because ~98% of PBMCs expressed the $T3^+$ $T4^{+/-}$ $T8^+$ phenotype, it appears conceivable that the proportion of normal lymphocytes was too low to give a positive MLR, the tumor cells themselves being unable to recognize the MHC-antigens on the stimulator cells.

Because the absolute number of Leu 11^+ cells was constantly increased, it appears possible that a third tumor cell subset existed. Since pan-T and suppressor/cytotoxic T cell epitopes were demonstrable on 98% of PBMCs, most Leu 11^+ cells probably reacted with anti-Leu 1, anti-Leu 4, and anti-Leu 2a as well, a possible hint to their T cell origin. Normally, only ~1.5% of PBMCs coexpress Leu 11 and Leu 2a, and no significant overlap is known to occur between Leu 11 and Leu 1, Leu 4, and Leu 3a.[16] Under physiological conditions, the Leu 11^+ subpopulation is functionally associated with ADCC and NK activity.[16] Our assays, however, failed to detect any cytotoxic activity of the patient's tumor cells.

Phenotypic heterogeneity of a tumor cell population is thought to reflect either the existence of multiple cell clones[43] or the retained capacity of a monoclonal cell line for further differentiation.[6] In our observation, several arguments are in favor of the latter assumption. First, cytogenetic analysis failed to demonstrate any chromosomally marked subclones within the tumor cell population. Second, regardless of the enormous variations of the WBCs, the ratio of Leu $2a^+$ Leu $3a^+$, Leu $2a^+$ Leu $3a^-$, and Leu 11^+ cells remained comparable throughout the observation period, suggesting that the tumor cell population as a whole responded to some regulatory mechanism; likewise the proportion of all subsets remained constant following initiation of antineoplastic chemotherapy. If multiple neoplastic cell clones had been present, such an observation could only be explained by the unlikely assumption of identical proliferation kinetics and identical sensitivity toward cytotoxic drugs of all clones. If a monoclonal tumor cell origin is hypothesized, the existence of multiple cell subsets could be explained either by different maturational stages on the way to a single terminal cell type, by multiple terminal cell types evolving from a common precursor, or by different functional states of the same cell population.[6] Although in our case both the $T3^+$ $T4^+T8^+$ and the $T3^+$ $T4^-$ $T8^+$ subset displayed the proliferation pattern of mature thymocytes,[22] it is conceivable that the $T4^+$ subset was the precursor of the $T4^-$ subset, as has been suggested with regard to other mature T cell neoplasms coexpressing T4 and T8.[41] However, our results do not exclude the possibility that both subsets originated from the same precursor with further differentiation into different paths. The ratio between Leu $2a^+$ Leu $3a^+$ and Leu $2a^+$ Leu $3a^-$ cells in our patient (56% to 69%:29% to 43%) reflected the ratio between helper/inducer and suppressor/cytotoxic T cells (47% to 78%:22% to 46%) in healthy individuals.[14] Similarly, the proportion of Leu 11^+ cells (3% to 9%) was comparable to the percentage of these cells (5% to 15%) under physiological conditions.[16] Although these similarities may be fortuitous, one is tempted to speculate that further differentiation of the incessantly proliferating tumor cells was controlled by regulatory mechanisms similar to those maintaining the physiological balance between the T cell subpopulations.

Chronic T cell leukemias have been reported in at least three other adult patients with AT (Tables 4 and 5). All cases showed comparable clinical findings. Apart from mild liver enlargement in two cases, organomegaly was not found at diagnosis. In spite of a more or less distinct bone marrow infiltration and a steadily increasing leukocytosis, bone marrow function remained sufficient for months or years. In the previously described cases, death resulted from respiratory failure either due to pleuropulmonary leukemic involvement in terminal stages (cases 1 and 3) or to bronchopneumonia facilitated by cytostatic therapy (case 2). The response to cytotoxic treatment was modest or absent; its tolerance was poor, with major complications arising from infections (cases 1 and 2) or cyclophosphamide-induced cystitis (present observation). A reduced tolerance to radiotherapy and chemotherapy is a well-known feature of AT,[44] possibly resulting from defective DNA repair.[45] An extensive phenotypic and functional analysis of the leukemic cells has only been done in the case described by Saxon et al.[6,7] Their findings reveal some resemblances to our own: The leukemic cells were subdivisible into two subpopulations carrying Fc-receptors for either IgM or IgG. Depending on the ratio

Table 4. Clinical Data of Patients with AT and T-CLL

Patient	Age (yr), Sex	Survival (mo)	Organ Involvement		Response to and Adverse Effects of Chemotherapy	Reference
			At Diagnosis	In Terminal Stages		
1	29, F	29	Bone marrow, liver?	Lymph nodes, liver, lungs, pleura (effusions), skin, hematopoietic insufficiency	Resistance to VCR + PRD; septic bursitis after CBL	[3,4]
2	25, F	14	Bone marrow	Mild hematopoietic insufficiency	Bronchopneumonia after CBL + PRD	[5]
3	47, F	42	Bone marrow, liver?	Pleura (effusions)	Resistance to CTX, CBL	[6–8]
4	27, M	24	Bone marrow	Pleura (effusions), mild hematopoietic insufficiency	Resistance to CBL + PRD, hemorrhagic cystitis after CTX + PRD	Present case

It is concluded from the literature that refs. 3 (case V) and 4 deal with the same patient.

AT, ataxia telangiectasia; T-CLL, chronic T cell leukemia; VCR, vincristine; PRD, prednisone; CBL, chlorambucil; CTX, cyclophosphamide.

Table 5. Laboratory Findings in Patients With AT and T-CLL

			Cellular Characteristics		
Patient	Diagnosis	WBC	Phenotype	Function	Karyotype
1	T-CLL	17,400–271,000	T: 81% B: 5%–8%	Response to PHA	41,XX, −13, −14, −15, −16, −20, 10q+, t(1;13), t(14;14), t(15;18)
2	Subacute T cell leukemia	48,400–613,000	T: 58% B: 11%	Response to PHA	43,XX, −13, −14, −16, −17, −21, −22, +3m, 18q+, t(13;15), t(13;17), t(14;14)
3	T-CLL	61,644–364,000	T: 73%–86% $\left.\begin{matrix}T_\gamma\\T\mu\end{matrix}\right\}$ increased B: <1%	Response to PHA, Con A; ADCC diminished; provision of help and suppression in immunoglobulin synthesis	44,XX, −14, −20, 6q−, i(8q), 12p−, 20p+, t(14;14)
4	T-CLL	52,500–881,100	see Table 1	see Tables 2 and 3	44,X, −Y, −20, 4q−, 6p−, 14q−, 19p+, 20q+, 22q−, t(4;20), t(6;19;22)

AT, ataxia telangiectasia; T-CLL, chronic T cell leukemia; PHA, phytohemagglutin; ConA, concanavalin A; ADCC, antibody-dependent cell-mediated cytotoxicity. WBC, white blood cell count in initial and terminal stages (cells/μL).

between leukemic T cells and normal B cells, the leukemic cells provided either help for or suppression of immunoglobulin synthesis in vitro. Response to PHA and ConA stimulation was normal; ADCC was diminished.

Complex clonal chromosome changes were found in all cases with T-CLL in AT, the unifying element being a structural anomaly of the long arm of chromosome 14. The three previously reported patients showed a tandem translocation t(14;14) involving band q11[5] or q12[3,8] of one chromosome 14 and band q31[8] or q32[3,5,40] of the other. Structural changes in bands q11–12 of chromosome 14 seem to occur in most chronic T cell neoplasms in patients with and without AT.[40,46] This chromosome region has recently been shown to code for the α-chain of the human T cell receptor[47,48] and is one of the "hot spots" for chromosome breakage even in lymphocytes of normal individuals.[49] Croce et al[47] state that malignant transformation in T-CLL may be related to juxtaposition of the α-chain gene locus to chromosome region 14q32.3, which they suspect to be the site of a proto-oncogene. Although our results do not exclude rearrangements at a molecular level, the karyotype in the present case differs from the previously reported observations: Whereas chromosome region q11–12 seemed to be conserved in both chromosomes 14, one chromosome 14 had an interstitial deletion affecting bands q21–31. Thus, it appears unlikely that the T cell receptor α-chain genes were involved in malignant cell transformation in our patient. Because the interstitial deletion concerned the region to which the *c-fos* oncogene has been mapped,[50] this oncogene may play a role in the pathogenesis of our case.

Clonal anomalies affecting chromosome 14 occur equally in lymphocytes of patients with AT not suffering from leukemia.[3,51-53] Because the proportion of these chromosomally marked cells tends to increase gradually with time,[3,51,52] one must consider the possibility that these patients will all sooner or later develop overt leukemia unless they die from other causes before. Various findings, however, support the view that a second event occurs, converting the "premalignant" state characterized by a sole anomaly of 14q into a neoplastic process.[54,55] First, although many cases with clonal anomalies of both chromosomes 14 have been followed up for several years,[3,51-55] only three patients with AT have been reported to develop T-CLL (apart from our observation).[3-8] Second, once T cell leukemia is diagnosed, deterioration is rather rapid in comparison to the benign clinical course before manifestation of lymphocytosis. Third, all cases with T-CLL and AT showed other complex chromosome anomalies in addition to those affecting chromosome 14.[3,5,8] Consistent cytogenetic abnormalities, however, have not been demonstrated yet in these patients. The malignant clones in the present case and in two of the three observations previously published[3,8] were marked by a loss of one chromosome 20, a finding that has repeatedly been observed in acute lymphoblastic leukemia.[56] However, the general significance of this aberration for the malignant transformation in AT appears questionable, because the same anomaly in connection with a rearrangement in chromosome 14 has been described in AT without leukemia.[55]

Chronic leukemias of phenotypically heterogeneous mature T cells may develop more often in AT than is yet known. To our knowledge, a comparable leukemia has never been described in patients without this hereditary disorder. Therefore, it is tempting to assume a genetic predisposition for the development of this particular type of T cell leukemia.

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This paper is dedicated to Prof Dr Karl Lennert on the occasion of his sixty-fifth birthday.

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Folate Requirements of Methotrexate-Resistant Human Acute Lymphoblastic Leukemia Cell Lines

By Yasuhiko Kano, Takao Ohnuma, and James F. Holland

We studied the folate requirements of a human acute lymphoblastic leukemia cell line, MOLT-3, and methotrexate (MTX)-resistant sublines established in vitro. The requirement of pteroylglutamate (PGA) for optimal cell growth was different for each cell line. With increasing MTX resistance, there was progressive increase in PGA requirements, moving the PGA concentration–cell growth curve (dose–response curve) 1 log order of magnitude to the right. The increases in the requirement of 5-methyltetrahydrofolate (5-methyl-THF) by the resistant sublines were more pronounced than PGA requirement, moving the dose-response curve nearly 3 log orders in magnitude to the right. The concentrations in vitro of 5-methyl-THF required for optimal growth of the MTX-resistant sublines far exceeded the normal serum 5-methyl-THF concentrations known in humans. These observations show that MTX-resistant cells established in vitro in culture media containing PGA instead of 5-methyl-THF, a physiological folate, cannot be expected to grow in vivo. The collateral sensitivity of transport-impaired MTX-resistant sublines to 2,4-diamino-5-methyl-6-[(3′,4′,5′-trimethoxyanilino) methyl] quinazoline (trimetrexate, TMQ) was negated in the absence of PGA. With the addition of 5-methyl-THF, the parent cells became more resistant than the transport-impaired sublines to TMQ. These data indicate that the collateral sensitivity of MTX-resistant cells to the substituted 2,4-diaminoquinazoline is due to functional folate deficiency by virtue of the impaired transport of folate.

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METHOTREXATE (MTX) is one of the most widely used agents for the treatment of leukemia, lymphoma, and other malignant diseases. Frequently, tumors that originally appeared to be responsive to MTX became increasingly resistant to this compound. The mechanism of acquired resistance to MTX has been studied in detail in a number of experimental systems including transplantable animal tumors in vivo and tumor cell lines in vitro. These studies revealed three major mechanisms for the development of MTX resistance: (a) an increase in dihydrofolate reductase activity[1-17]; (b) a decrease in the affinity of dihydrofolate reductase to MTX[8,9,13,18,19]; and (c) a decrease in the MTX transport through the cell membrane.[5,6,8,12-15,17,18,20-23] As a fourth mechanism of MTX-resistance, the inability of cells to convert the drug to its polyglutamate derivatives has recently been suggested.[24] Among them, the increase in dihydrofolate reductase and the decrease in membrane transport have been considered as the major mechanisms of MTX-resistance, since a majority of MTX-resistant cells established, mainly in vitro, had either of them or both. When MTX-resistant cells were established in vivo, however, in only a few isolated cases has impaired-transport been recognized as the mechanism of resistance.[5,15] At the clinical level, no reports have indicated impaired transport of MTX as the mechanism of an acquired resistance. In cases of acquired resistance, an elevated dihydrofolate reductase or a decreased affinity of the enzyme to MTX was demonstrated.[4,7,10,16,19,25]

The in vitro culture condition is different from the in vivo condition in that commercial media used for maintaining cells or establishing MTX-resistant cells contain pteroylglutamate (PGA), an oxidized form of folic acid, instead of 5-methyltetrahydrofolate (5-methyl-THF), a physiological folate. It is known that MTX shares the common carrier-mediated process with reduced folates, 5-methyl-THF, and 5-formyltetrahydrofolate (5-formyl-THF), to enter cells,[15,20,22,26,27] and the transport of these reduced folates is decreased in transport-impaired MTX-resistant cell lines.[15,17,20,22,23,27] Although the nature of the PGA transport system is not precisely understood, a major portion of PGA was shown not to use a reduced-folate–MTX-transport system to enter cells.[20,26,28] Thus, it is possible that although transport-related MTX-resistant cells grow avidly in vitro, they may not be able to take up sufficient quantities of 5-methyl-THF for optimal cell growth in vivo.

We examined the folate requirements of MTX-resistant sublines of a human acute lymphoblastic leukemia cell line, MOLT-3, established in vitro. We also compared the effect of 5-methyl-THF on the sensitivity of these cells against MTX and 2,4-diamino-5-methyl-6-[(3′,4′,5′-trimethoxyanilino)methyl] quinazoline (trimetrexate, TMQ).

MATERIALS AND METHODS

Cell lines. The cell lines used were human acute lymphoblastic leukemia cell line MOLT-3[29] and 3 MTX-resistant sublines.[17] The sublines were established in our laboratory and were maintained as a suspension in culture flasks containing RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO) and antibiotics (penicillin G 100 U/mL and streptomycin 100 μg/mL).

Sublines MOLT-3/MTX$_{30}$, MOLT-3/MTX$_{200}$, and MOLT-3/MTX$_{10,000}$ were 30, 200, and 10,000 times more resistant to MTX, respectively, as compared with the parent MOLT-3 cells when tested in RPMI 1640 medium plus 10% FBS or in McCoy's 5a medium devoid of PGA and vitamin B$_{12}$ (Nissui Seiyaku, Tokyo) that was supplemented with 20% FBS plus 2.3×10^{-6} PGA (Sigma, St Louis) plus 2 mg/L vitamin B$_{12}$(Sigma). MOLT-3/MTX$_{30}$ and

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MOLT-3/MTX$_{200}$ have impaired transport of MTX and 5-methyl-THF and no significant elevation of dihydrofolate reductase activity. MOLT-3/MTX$_{10,000}$, derived from MOLT-3/MTX$_{200}$, has both impaired transport of MTX and elevated dihydrofolate reductase activity. Data regarding the doubling time, MTX and methyl-THF transport, and dihydrofolate reductase activity of these cells are summarized in Table 1.[17]

Cell maintenance and preparation. All cell lines originally maintained in RPMI 1640 medium plus 10% FBS plus antibiotics were transferred to the special McCoy's 5a medium (devoid of PGA and vitamin B_{12}) supplemented with 20% FBS, vitamin B_{12}, and PGA. PGA concentrations used were 2.3×10^{-6} mol/L for MOLT-3 and MOLT-3/MTX$_{30}$, and 1×10^{-5} mol/L for MOLT-3/MTX$_{200}$ and MOLT-3/MTX$_{10,000}$. A larger concentration of PGA was required for the optimal cell growth of MOLT-3/MTX$_{200}$ and MOLT-3/MTX$_{10,000}$(Table 1).

Folate requirement studies. Logarithmically growing cells were harvested, washed three times in the special McCoy's 5a medium devoid of PGA and vitamin B_{12}, and resuspended to a final concentration of 1×10^6 cells/10 mL of the medium containing 20% dialyzed FBS and vitamin B_{12} with or without PGA. Fifteen milliliters of FBS were dialyzed three times at 4 °C for 24 hours against 1 L of a solution containing 8 g of NaCl, 0.4 g of KCl, 1 g of glucose, and 0.35 g of $NaHCO_3$, and used as dialyzed FBS.

Initially, in order to determine the time period required to exhaust intracellular folate pools, cell growth in the media with or without PGA was studied for seven days. Ten-milliliter aliquots (10^6 cells) of the cell suspensions with or without PGA were placed in a tissue culture flask (Falcon, Oxnard, Calif, No. 3033) and incubated in 5% CO_2 in humidified air at 37 °C for three days. The cells were then counted with an improved Neubauer hemocytometer. Cell viability was defined as the ability of cells to exclude trypan blue dye (GIBCO). The cells were then diluted to a final concentration of 1×10^6 cells/10 mL in the same fresh medium and incubated until day 7. Viable cells were enumerated on days 5 and 7.

For the study of folate requirements, the cells were grown for three days in the medium devoid of PGA. These cells were then harvested from the medium and resuspended to a final concentration of 1×10^5 cells/mL in the same fresh medium. One-milliliter aliquots of cell suspensions were placed in individual wells of a 24-well tissue culture plate (Falcon No. 3047). 5-Methyl-THF (Sigma), 5-formyl-THF (Lederle Laboratories, Pearl River, NY), and PGA were freshly dissolved in Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (GIBCO) containing sodium ascorbate (5 mg/mL, Sigma), and were added in volumes of 10 μL to each culture well. They were incubated further in 5% CO_2 in humidified air at 37 °C for four days, and viable cell growths were determined.

Table 1. Characteristics of Human Acute Lymphoblastic Cell Line, MOLT-3, and Its MTX-Resistant Sublines

	Doubling Time (h) PGA Concentration (M) in Culture Medium		Influx (V_{max})* of		Dihydrofolate Reductase Activity	
	2.3×10^{-6}	1.0×10^{-5}	MTX	5 methyl-THF	MTX-Binding Assay†	Dihydrofolate Assay‡
MOLT-3	24	24	7.7	6.7	18	15
MOLT-3/MTX$_{30}$	25	24	1.7	5.7	20	22
MOLT-3/MTX$_{200}$	32	27	1.4	3.7	20	14
MOLT-3/MTX$_{10,000}$	36	29	1.1	3.3	210	85

*nmol/2.5 minute per 10^7 cells. K_m is constant at ~6.6 μmol/L for both MTX and 5-methyl-THF.

†pmol of MTX bound per 10^8 cells.

‡nmol of dihydrofolate reduced per minute per 10^8 cells.

Data on doubling time represent the mean of at least three measurements. Data on influx represent a mean of three separate experiments carried out in duplicate (modified from Ref. 17). Data on dihydrofolate reductase are representative values from longitudinal studies. Results are expressed as a mean of an assay done in triplicate. Range of the values in triplicate determination was ± 10% of the mean.[17]

In this condition, 5-formyl-THF and PGA are stable, whereas 5-methyl-THF loses 30% of its activity after two days of incubation as determined by bioassay using *L casei, S faecalis, and P cerevisiae* (H. Taguchi, and Y. Kano, unpublished observations).

TMQ sensitivity. Cell suspensions prepared as above in the absence of PGA were dispensed (1 mL) into individual wells of the 24-well tissue culture plate. Ten microliters each of graded concentrations of 5-methyl-THF were added to each well. TMQ (obtained through the National Cancer Institute, Bethesda, Md) dissolved in pure ethanol was further diluted with Dulbecco's PBS without calcium and magnesium, and was added to each culture well. They were incubated in 5% CO_2 in humidified air for three days, and viable cells were enumerated. TMQ sensitivity of these cell lines were also studied in RPMI 1640 medium plus 10% FBS. Each experiment was performed in duplicate or triplicate and was repeated two or more times for each cell line.

RESULTS

Folate requirements. As expected, all of the cell lines grew well in the medium containing PGA. In the McCoy's 5a medium with 20% dialyzed FBS and devoid of folate, however, the cells grew avidly for the first three days and then cell growth gradually slowed. Therefore, cells incubated without PGA for three days were used for the study of PGA, 5-methyl-THF, and 5-formyl-THF requirements for cell growth. The effects of various concentrations of PGA and 5-methyl-THF on the cell growth of MOLT-3 and MTX-resistant sublines are illustrated in Figs 1 and 2.

PGA concentrations required for optimal cell growth were

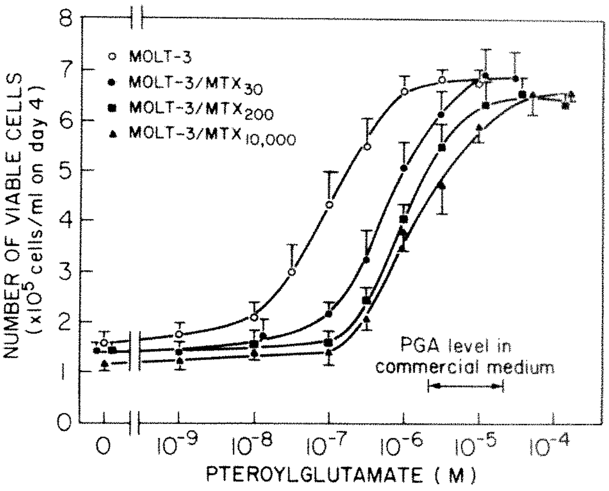

Fig 1. Pteroylglutamate (PGA) requirements in MOLT-3 and MTX-resistant sublines. Cells that had been maintained in McCoy's 5a medium without PGA for three days were resuspended in the same fresh medium to a density of 1×10^5 cells/mL, and a graded concentration of PGA was added. Cells were grown for an additional four days, and the viable cell number was determined. Values are mean ± SD. PGA concentrations in commercial culture media are shown in right lower corner.

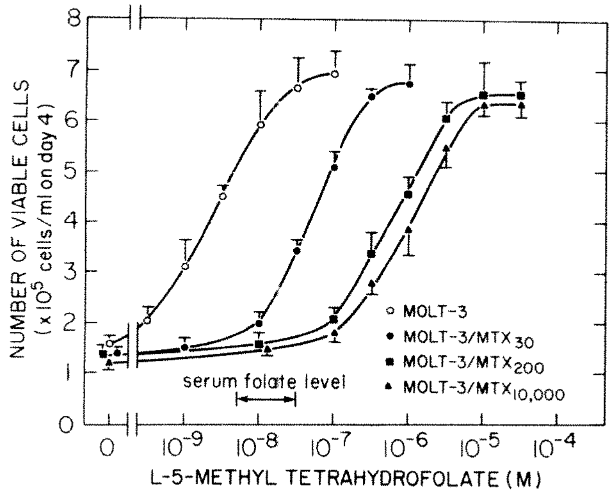

Fig 2. 5-Methyltetrahydrofolate requirements in MOLT-3 and MTX-resistant sublines. Values are mean ± SD. Details as in Fig 1. Reported normal range of serum folate in humans is shown in the lower portion of the graph. Most physiological folates are in the form of 5-methyltetrahydrofolate.

different for each cell line (Fig 1). With increasing MTX resistance PGA requirements progressively increased, moving the drug concentration–cell growth curve (dose–response curve) to the right. The maximal move of the dose–response curve was ~1 log order in magnitude. The most MTX-resistant cell line, MOLT-3/MTX$_{10,000}$, required 1.1×10^{-6} mol/L of PGA for 50% optimal cell growth, and the PGA concentration for optimal cell growth was $\geq 1 \times 10^{-5}$ mol/L. These values can be contrasted with PGA concentrations in commercially available media [eg, 2.3×10^{-6} mol/L and 2.3×10^{-5} mol/L for RPMI 1640 medium (GIBCO) and McCoy's 5a medium (GIBCO), respectively]. These data indicate that PGA concentrations in these media are well above the concentration required for the optimal growth of the parent MOLT-3 cells. MOLT-3/MTX$_{200}$ and MOLT-3/MTX$_{10,000}$ cells must have been maintained at threshold or suboptimal concentrations of PGA for cell growth.

Changes in the 5-methyl-THF requirements for the optimal cell growth of the resistant sublines were more pronounced, moving the dose–response curve nearly 3 log orders in magnitude to the right (Fig 2). The changes in the 5-methyl-THF requirements appeared to be parallel to the degree of acquired resistance to MTX for the purely transport-related sublines. Thus, the folate concentration required for 50% optimal cell growth increased 19-fold, 219-fold, and 407-fold of the parent cells for MOLT-3/MTX$_{30}$, MOLT-3/MTX$_{200}$, and MOLT-3/MTX$_{10,000}$, respectively.

The normal serum folate concentration in humans is reported to be in a range from 5×10^{-9} to 3×10^{-8} mol/L, with most folate in the form of 5-methyl-THF.[30,31] The concentrations in vitro of 5-methyl-THF required for optimal cell growth in the MTX-resistant sublines (3×10^{-7} to 1×10^{-5} mol/L) far exceeded the normal serum 5-methyl-THF levels in humans. These observations indicate that highly transport-impaired MTX-resistant cells cannot be expected to grow in vivo.

A similar move in the dose–response curve (~3 log orders in magnitude) was also seen as the requirement of 5-formyl-THF with increasing MTX resistance (data not shown). To produce identical biologic effects, 5-formyl-THF requirements were, on a molar basis, threefold to fourfold less than that of 5-methyl-THF.

TMQ sensitivity. Comparative inhibitory effects of TMQ on the parent and MTX-resistant sublines maintained in RPMI 1640 medium containing 10% FBS are shown in Fig 3. Transport-impaired MTX-resistant sublines were found to be more sensitive to TMQ than the parent line was.[17,23] Thus, the TMQ concentration required for 50% inhibition of cell growth on day 3 (ID$_{50}$) was 1.8×10^{-8} mol/L for the parent cell lines and 5.0×10^{-9} and 4.0×10^{-9} mol/L for MOLT-3/MTX$_{30}$ and MOLT-3/MTX$_{200}$ cell lines, respectively. MOLT-3/MTX$_{10,000}$ was found to be as sensitive as the parent line to TMQ to the point of ID$_{50}$; however, the dose–response curve approached a plateau at higher TMQ concentrations, indicating the presence of highly TMQ-resistant cell populations. Their TMQ sensitivities in McCoy's 5a medium plus 20% dialyzed FBS containing various concentrations of 5-methyl-THF are illustrated in Fig 4. Without 5-methyl-THF, the TMQ sensitivity was virtually identical for MOLT-3, MOLT-3/MTX$_{30}$, and MOLT-3/MTX$_{200}$. In the absence of 5-methyl-THF, however, MOLT-3/MTX$_{10,000}$ was found to be more resistant than the other cell lines. Progressive increases in 5-methyl-THF concentrations resulted in a marked shift of the drug concentration–inhibition curve (dose–response curve) of the parent cells. In contrast, the shift of the dose–response curve to TMQ was progressively less influenced with increasing MTX-resistance.

DISCUSSION

The major aim of the present study was to evaluate whether the mechanism of MTX-resistance and collateral

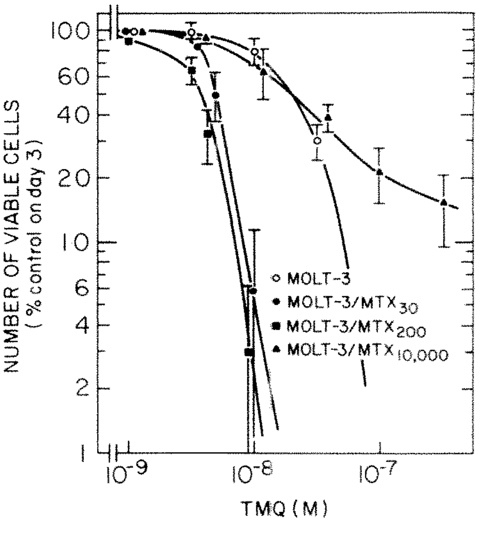

Fig 3. TMQ sensitivity of MOLT-3 and MTX-resistant sublines in RPMI 1640 medium containing 10% fetal bovine serum. Cells were cultured for three days; viable cells were enumerated and expressed in percentage as compared with those in control wells without drugs. Values are mean ± SD.

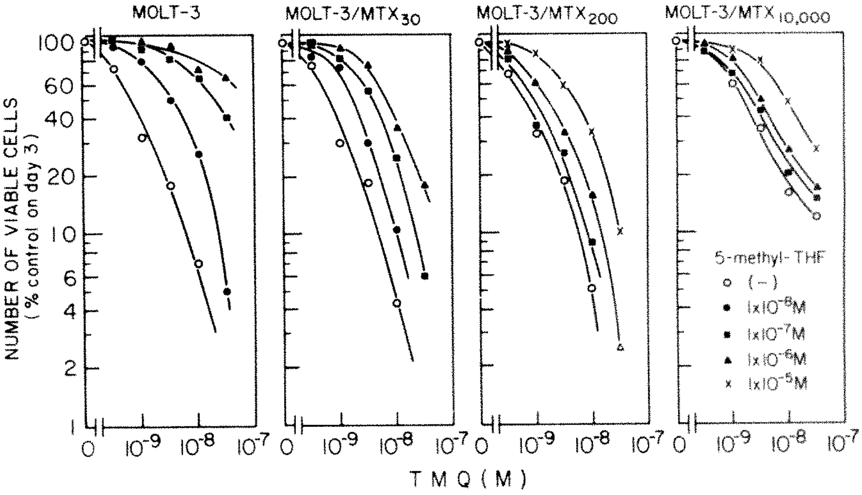

Fig 4. TMQ sensitivity of MOLT-3 and MTX-resistant sublines in McCoy's 5a medium containing 20% dialyzed FBS and graded concentrations of 5-methyl-tetrahydrofolate (5-methyl-THF). Details as in Fig 3.

sensitivity of MTX-resistant cells to TMQ, both demonstrated in vitro, could be operational in vivo.

MOLT-3/MTX$_{30}$, MOLT-3/MTX$_{200}$, and MOLT-3/MTX$_{10,000}$ cells required much higher levels of 5-methyl-THF, 5-formyl-THF, and PGA than did the parent MOLT-3 cells. For optimal growth, they required at least 3.0×10^{-7} mol/L, 3.0×10^{-6} mol/L, and 1.0×10^{-5} mol/L 5-methyl-THF, respectively. A similar increase was observed for 5-formyl-THF requirements. At physiological levels of 5-methyl-THF, MOLT-3/MTX$_{30}$ may grow at a substantially reduced rate; however, these concentrations of reduced folate were unable to support cell growth for the highly MTX-resistant sublines. These data indicate that highly transport-impaired MTX-resistant MOLT-3 cells cannot be expected to grow in vivo.

Although 5-methyl-THF and 5-formyl-THF are considered to use the same transport system to enter the cells, we found that 5-methyl-THF requirements were three to four times higher than those of 5-formyl-THF. The reasons for this discrepancy include the instability of 5-methyl-THF during four days of incubation and the difference in the entry site of these two reduced folates in the metabolic pathway. Our observations are consistent with reports that 5-formyl-THF corrected folate deficiency in hematopoietic cells more effectively than did 5-methyl-THF in short-term culture.[32,33]

The commercial media contained nonphysiological and stable PGA instead of physiological and unstable folate, 5-methyl-THF. The increased requirements of PGA were observed in MTX-resistant MOLT-3 sublines; however, the increases were only 5-fold to 11-fold. Therefore, even if MOLT-3 cells lost their capacity to transport reduced folates into the cell during the process of acquiring MTX-resistance, these cells can still grow in a medium containing PGA.

An increase in the doubling time of the resistant sublines in RPMI 1640 medium plus 10% FBS and the reversal by increasing PGA concentrations (Table 1) indicate that the PGA concentration in the commercial RPMI 1640 medium plus 10% FBS is not adequate for their optimal growth.

The nature of the concomitant shift in the requirements of PGA and reduced folates in MTX-resistant sublines is not clear. PGA has been considered not to use a reduced folate-MTX transport system.[20,26,28] The changes in the membrane structure or the transport protein for the entry of reduced folate-MTX may have influenced the entry of other nutrients including PGA. It is difficult, however, to rule out the possibility of a much closer tie between the transport systems of PGA and MTX.

We observed that in the absence of folate in the medium, the degree of TMQ sensitivity was virtually identical among parent and transport-impaired MTX-resistant sublines. The presence of 5-methyl-THF produced a lesser influence on the shift of the TMQ dose–response curves of sublines with higher MTX-resistance, resulting in apparent higher sensitivity to TMQ for the resistant sublines as compared with the parent cells at identical 5-methyl-THF concentrations. These data indicate that the collateral sensitivity of transport-impaired MTX-resistant sublines to TMQ is related to their impaired folate transport. Our observation confirms the statement by Sirotnak et al that collateral sensitivity to lipophilic antifolates in MTX-resistant animal cells is probably attributable to relatively low levels of intracellular folate by virtue of impaired transport of folate.[15]

We find that MOLT-3/MTX$_{10,000}$ cells, which have both impaired transport of MTX and an elevated dihydrofolate reductase, are more resistant to TMQ in the absence of folic acid (Fig 4). This finding is in accord with the dose–response curve of MOLT-3/MTX$_{10,000}$ seen in Fig 3. It may be concluded, therefore, that MTX-resistant cells with elevated dihydrofolate reductase have cross-resistance with lipophilic antifols. This observation is consistent with a report that lipophilic antifolates are not active on MTX-resistant cells with elevated dihydrofolate reductase.[15,17]

Although transport-resistant cells thus obtained in vitro may serve as a useful tool in the study of membrane transport of nutrients, our data cast strong doubts as to the development of a high degree of transport resistance as the primary cause of acquired MTX resistance in the clinical setting. Bertino et al reported that dihydrofolate reductase activity was found to be elevated in leukemic cells from patients treated with MTX.[4,7] Some MTX-resistant–leukemic patients also had a low affinity of MTX binding to dihydrofolate reductase.[19] More recently, Curt et al established a

MTX-resistant tumor cell line derived from a patient with small cell lung cancer who had been treated with MTX and had become resistant to MTX.[16] This cell line displayed an elevation of dihydrofolate reductase accompanied by amplification of the gene coding for the enzyme. In addition, Hoffbrand et al reported that there was no difference in MTX transport between MTX-sensitive and MTX-resistant leukemic cells obtained from patients with acute myelogenous leukemia (AML).[25] Therefore, no clinical case has demonstrated impaired MTX transport as the primary cause of acquired MTX resistance. This is in sharp contrast to the report of Kessel et al demonstrating that MTX transport is a critical determinant of clinical responses to MTX in human leukemias.[34] Their work indicates that decreased MTX-transport is a major cause of natural resistance to MTX. Although Sirotnak et al established MTX-resistant L1210 cells by virtue of impaired transport of MTX in transplantable animals,[15] the resistance was only about tenfold as compared with that of the parent cells. We believe that in transplanted animals, or even in clinical cases, a mild degree of MTX-resistance may occur by virtue of mildly impaired MTX transport alone. In such cases, the impairment in reduced folate transport is to such a degree that sufficient quantities of reduced folate for optimal cell growth can still be incorporated into the cells. In highly MTX-resistant cells, the elevation in dihydrofolate reductase appears a more likely mechanism of acquired MTX resistance in vivo.

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CONCISE REPORT

The Structure of the T Cell Gamma Chain Gene in Lymphoproliferative Disorders and Lymphoma Cell Lines

By H. Griesser, A. Feller, K. Lennert, M. Tweedale, H.A. Messner, J. Zalcberg, M.D. Minden, and T.W. Mak

During the development of B and T cells, a number of genes undergo rearrangement. In this paper we have studied the structure of the T cell γ-chain gene in primary lymphomas and cell lines derived from patients with lymphoma. Samples derived from patients with angioimmunoblastic lymphadenopathy (AIL), Lennert's lymphoma, and Hodgkin's disease were also examined. TcRγ was rearranged in all T cell lymphomas and B cell lymphoma cell lines. Rearrangement of the γ-chain gene was also found in AIL, Lennert's lymphoma, and four of eight cases of Hodgkin's disease. These studies indicate that rearrangement of TcRγ is a useful clonal marker but does not aid in the identification of cell lineage.

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DURING THE DEVELOPMENT of B cells and T cells, a number of genes undergo somatic rearrangement. In the B cell, these are the immunoglobulin (Ig) light (LC) and heavy chain (HC) genes,[1] and in the T cell, the T cell antigen receptor (TcR) β-, α-, and γ-genes.[2-4] Rearrangements are random so that the structure of the resulting gene varies widely from cell to cell. In B and T cell malignancies, clonal expansion of a single cell results in all members of the clone having the same rearrangement. Due to the limited sensitivity of Southern blot analysis,[5,6] it is possible to distinguish clonal populations of lymphocytes from nonclonal populations of lymphocytes. In addition, the genes that are rearranged in the tumor cells give some indication as to the lineage of the cell.[1,2] Through the study of tumor cells and tumor cell lines, the order in which genes are rearranged during differentiation has been determined.[1] For example, in the B cell lineage, Ig HC rearrangement is followed by κ LC rearrangement, which in turn may be followed by λ LC rearrangement.

In T cells, three genes undergo somatic rearrangement; however, the order in which this occurs and the specificity of rearrangement in defining the lineage of a cell are not well established. Previously, we studied a number of functional T cell clones and leukemic T cell lines and found that in cases in which there was rearrangement of the TcRβ chain gene there was also rearrangement of the γ-chain gene; cells with rearranged γ but not β-chain genes were not identified.[7] In order to determine whether this was the case in other malignant cells of T cell origin and to test the specificity of γ-chain gene rearrangement for the T cell lineage, we have examined DNA derived from involved lymph nodes of patients with lymphoproliferative disorders and B cell lymphoma cell lines. These studies indicate that rearrangement of TcRβ occurs before TcRγ and that rearrangements of the γ-chain locus by itself is a poor indicator of cell lineage.

MATERIALS AND METHODS

Patient material. Lymph nodes of individuals undergoing investigation of lymphoma were snap frozen and stored at −70 °C.[8] The lymphomas were characterized by routine histochemistry and immunochemistry as previously described.[9] There were 14 cases of T cell lymphoma (two lymphoblastic lymphoma and 12 peripheral T cell lymphoma); seven cases of B cell lymphoma (one B cell chronic lymphocytic leukemia, one centrocytic lymphoma, one centroblastic/centrocytic lymphoma, two immunoblastic lymphoma and two cases with monotypic B cells and a large proportion of pleomorphic T cells); five cases of Lennert's lymphoma; eight cases of Hodgkin's disease; ten cases of non-Hodgkin's lymphoma containing large anaplastic Ki 1$^+$ cells (Ki 1$^+$ lymphoma) (ten); nine cases of AIL; and two cases of AIL-like hyperimmune reaction.

Cell lines. Recently, eight cell lines derived from patients with non-Hodgkin's lymphoma have been established at the Ontario Cancer Institute. All of the cell lines are Epstein-Barr virus negative and express surface or cytoplasmic immunoglobulin.[11]

Analysis of DNA. DNA was extracted from the lymph nodes or cell lines as previously described[8] and digested with one of the restriction enzymes *Bam*HI, *Eco*RI, or *Hind*III, according to the suppliers' recommendations. The cut DNA was separated by electrophoresis on a 0.8% agarose gel, transferred to nitrocellulose according to the method described by Southern, and hybridized to a ^{32}P-labeled cDNA[3,12] containing the γ-gene (a gift from Dr D. Littman, University of California, San Francisco). Rearrangement was scored by the loss of the germline band or the appearance of new bands.

RESULTS

The results of the Southern blot analysis using a γ-cDNA probe are summarized in Table 1, and representative autoradiographs shown in Fig 1. Rearrangement of TcRγ chain gene resulted in the appearance of new bands and variable loss of the germline band. Rearrangement of TcRγ gene was found in 14 of 14 cases of T cell lymphoma (Fig 1A); six of seven B cell lymphomas; five of five cases of Lennert's lymphoma; eight of eight Ki 1$^+$ lymphomas; nine of nine cases of AIL; and four of eight cases of Hodgkin's disease

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Table 1. Structure of Rearranging Genes in Lymphohematopoietic Malignancies

Diagnosis	No. of Patients	Germline	Ig HC + LC	Ig HC + TcRγ	Ig HC + LC + TcRγ	Ig HC + LC + TcR β + γ	TcRγ	TcR β + γ	TcRβ
T cell lymphoma	14							14	
B cell lymphoma	7		1	1	3	2			
B cell lymphoma cell lines	8		5		3				
Lennert's lymphoma	5							5	
Ki 1⁺ lymphoma	8			3			2	3	
Angioimmunoblastic lymphadenopathy	11	2*		3	1			5	
Hodgkin's disease	8	4						4	

*These two cases were diagnosed as AIL-like hyperimmune reaction.[20]

(Fig 1b). Rearrangement was not seen in two cases of AIL-like HR.

In the B cell lymphoma cell lines, rearrangement of TcRγ chain was found in three of the eight cell lines.

A number of patterns of rearrangement were seen; however, within and between different classes of tumors, similar rearranged bands could be seen. This is illustrated in Fig 1A, in which lanes 2, 3, 6, 7, and 11 all contain an apparently identical rearranged band.

DISCUSSION

In the present study we have examined the structure of the T cell γ-chain gene in 55 primary lymphomas and eight lymphoma cell lines. The structure of the Ig LC and HC genes and the TcRγ chain gene of the lymphoma samples have been reported; those results are summarized in Table 1.[8] Similar studies have been carried out on the lymphoma cell lines, and the results are also presented in Table 1 (manuscript in preparation). As can be seen in Table 1, rearrangement of TcRγ chain gene may occur in a number of settings.

In agreement with our previous studies, in all cases in which there was rearrangement of TcRβ, there was also rearrangement of TcRγ. However, in the present study we also found TcRγ rearrangements in association with rearranged Ig HC and LC genes; this could be with or without TcRβ rearrangement. Furthermore, two cases were identified in which there was TcRγ rearrangement in the absence of Ig or TcRβ rearrangement.

The two cases in which there was only TcRγ rearrangement were cases of Ki 1⁺ lymphoma. The Ki 1 antigen is detected by a monoclonal antibody raised against a Reed-Sternberg cell line.[13] The antibody reacts with Reed-Sternberg cells, activated normal T lymphocytes, and almost all of the cells in some large anaplastic lymphomas; the latter are designated Ki 1⁺ lymphomas.[10] Immunohistochemical studies indicate that these lymphomas are mainly of T cell origin, occasionally of B cell origin, and seldom of histiocytic origin. In a previous report we suggested that the two cases in which there was no clonal rearrangement of the Ig and TcRβ genes may be a polyclonal proliferation of lymphocytes. However, the detection of a rearranged γ-chain gene indicates that they are clonal in origin.

The nature of the cell in the Ki 1⁺ lymphomas that only have TcRγ rearrangement is unknown. Whether it represents an early lymphocyte precursor that became frozen in differentiation as a result of malignant transformation[14] or is the malignant counterpart of a mature functional cell type not yet recognized will be resolved by more intensive study of the cell involved in such lymphomas.

Rearrangement of the γ-chain gene was also found in a large proportion of B cell lymphomas and B cell lymphoma cell lines. These tumor cells have rearranged their immuno-

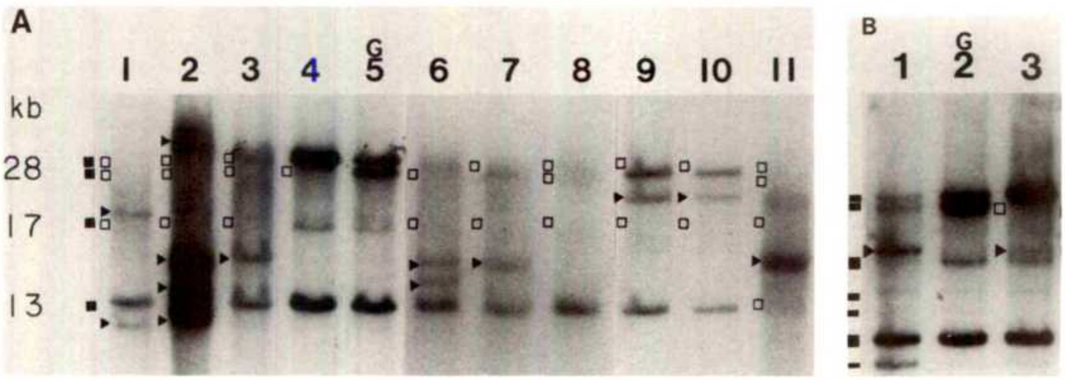

Fig 1. Southern blot analysis of DNA cut with *Bam*H1 and probed with ^{32}p-labeled gamma chain cDNA. (A) DNA derived from ten T cell lymphomas. (B) DNA from two cases of Hodgkin's lymphoma. The lane G in each sample is DNA derived from granulocytes and represents the germline pattern. ■, germline bands; ▲, rearranged bands; □, deleted germline bands.

globulin light and heavy chain genes and express either cytoplasmic or surface Ig. In some of these cases, γ-gene rearrangement was present in the absence of TcRβ rearrangement.

These findings, along with our previous studies of T cell leukemia cell lines and functional T cell clones and studies in mouse,[16] suggest that rearrangement of the γ-chain gene is an early event in the development of lymphocytes. The present data are consistent with the following developmental scheme. In the development of a functional T cell, rearrangement of TcRβ occurs only if the γ-chain gene has rearranged. In the development of a functional B cell prior to rearrangement of the γ-gene, neither appears to be essential for the subsequent rearrangement of the HC genes nor does it restrict a cell from differentiating along the B cell pathway. However, the frequent association of γ-chain rearrangement with Ig rearrangement in mature B cells suggests that early events involved in lymphocyte differentiation that affect DNA conformation[17] and recombinase activity[18] are relatively indiscriminate and can act on Ig HC, TcRγ, and TcRβ chain genes. Second, the association of Ig and TcRγ rearrangements in the same B cell suggests that these rearrangements are occurring in the bone marrow. This is in contrast with other studies suggesting that TcRγ rearrangement occurs in the thymus.[16]

The finding of gamma rearrangement in lymph nodes from patients with Hodgkin's disease confirms our previous observation of a clonal population of cells in some cases of this disease. Studies are now in progress to determine whether this finding can identify a subset of patients with regard to histologic subtype or clinical behavior. One of the samples contained 50% Reed-Sternberg cells; in this case no rearrangements were detected.

In Fig 1, new bands of different molecular weights are apparent. However, similar size bands are also present in different samples. This observation persists even when different restriction enzymes are used. A possible reason for this is the limited repertoire of TcRγ chain gene. Analysis of γ cDNA clones reveals that there are, at most, ten variable regions and six joining regions.[19] However, enough variability in the size of the rearranged fragment exists so that in a polyclonal population of T cells, such as obtained from peripheral blood, rearrangement is not evident.

As rearrangement of TcRγ chain gene may be associated with TcRβ rearrangement or Ig rearrangement, it is not a useful marker of lineage. However, since there appears to be a range of possible rearrangements of the gamma chain gene, it should prove to be a useful marker of clonality of both T and B cell malignancies. In addition, a new cell type having only gamma gene rearrangement may have been identified.

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CORRESPONDENCE

EXPRESSION OF PLATELET GLYCOPROTEIN IIIa BY HUMAN OSTEOCLASTS

To the Editor:

In a recent article in *Blood*, Beckstead et al[1] reported the immunological localization of a series of membrane and granule proteins by human megakaryocytes in bone marrow biopsy specimens. An unexpected finding in this study was the observation that platelet glycoprotein IIIa (GPIIIa), but not GPIIb or GPIIb/IIIa complex, was expressed by human osteoclasts. We have previously studied the cell surface expression of a range of hemopoietic antigens by human fetal[2] and tumor-associated[3] osteoclasts. In an extensive survey we found that osteoclasts were HLA-DR negative and failed to express myeloid antigens; the only exception was reactivity with My7-like antibodies, which recognize a 150-kd surface glycoprotein present on the majority of myeloid cells. Further, we have recently raised a series of monoclonal antibodies to human osteoclasts.[4] These fail to react with megakaryocytes in human[4] and animal[5] bone marrow.

We report here our results on the expression of platelet-associated determinants by human osteoclasts isolated from giant cell tumors of the bone (osteoclastomas).[3,4] Osteoclasts are GPIIIa (C17[6]) positive (Fig 1) but fail to react with antibodies to GPI (AN51), GPIIb/IIIa (J15), GPIIb (P256), platelet factor 4, or factor VIII-related antigen, confirming the findings of Beckstead et al.[1]

The coexpression of GPIIIa by megakaryocytes, platelets, and osteoclasts clearly needs further investigation. It would be of particular interest to know if GPIIIa mediated some functional role, such as cellular adhesion, shared among these diverse cell types.

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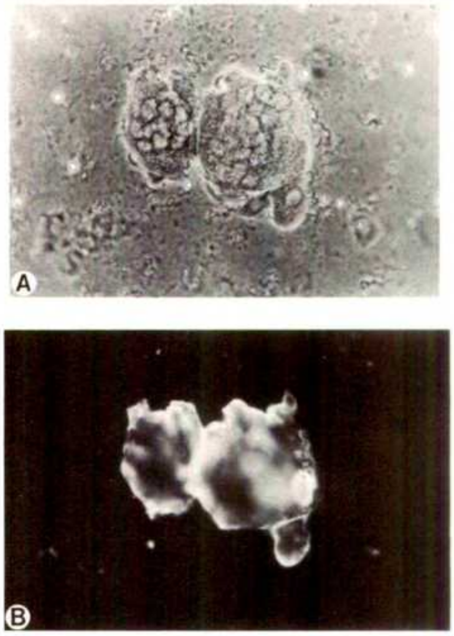

Fig 1. Phase (A) and fluorescence (B) migrographs of giant cell bone tumor osteoclasts reacted with monoclonal antibody C17 to platelet GPIIIa.

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FOLLICLE LYSIS IN LYMPH NODES FROM HOMOSEXUAL MEN

To the Editor:

We read with interest the article by Wood et al[1] about the "follicle lysis" phenomenon, which is due to the disruption of the follicular dendritic cell (FDC) framework[2] in lymph nodes from homosexual men with persistent generalized lymphadenopathy (PGL). Because FDCs express the T4 antigen, the authors suggest that their infection by HTLV III/LAV may play a role in the pathogenesis of follicle lysis.

We agree with them. As is known, viral particles in close contact with the cytoplasmic processes of dendritic reticulum cells (DRC) were seen by Tenner-Racz et al[3] and Armstrong et al.[4] We were able to demonstrate[5] the strong intracytoplasmic reactivity of the FDC (Fig 1) from PGL and AIDS patients using the CVK monoclonal antibody specific for the P18/LAV protein[6] and modified immunoperoxidase technique. No cells other than FDC were stainable in lymph nodes with CVK, despite the fact that the T4 antigen is demonstrable on other cells in lymph nodes.[7]

These results suggest that FDCs represent the elective target of HTLV III/LAV infection in lymph nodes. However, the progressive disruption of the FDC framework, with follicle lysis, is the main morphological aspect of lymph nodes in PGL patients. We have no certain data about the mechanisms involved in such a phenomenon; perhaps the entrance of T8 cells into the follicles[8] could implicate cell-mediated damage of infected DRC rather than a direct cytopathic effect of the virus.

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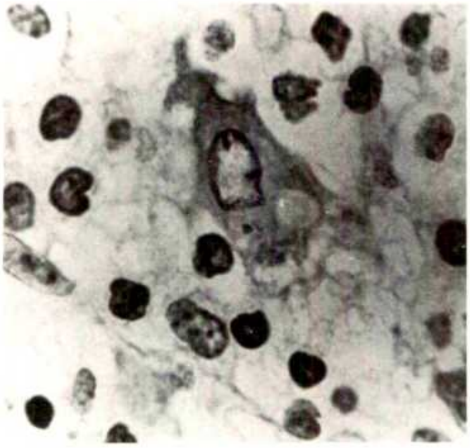

Fig 1. Lymph node biopsy from patient with persistent generalized lymphadenopathy. Two-micron sections from historesine embedded biopsy immunostained with P18/LAV MoAb, showing strong intracytoplasmic reactivity in the follicular dendritic cell.

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To the Editor:

We read with great interest the letter by Parravicini et al, who report FDC reactivity with a monoclonal antibody directed against p18/LAV protein. As these investigators have noted, their results are consistent with prior immunohistologic[1,2] and ultrastructural studies[3,4] suggesting that, in addition to helper T cells, FDC may be a target of HTLV-III/LAV infection. One basis for this hypothesis is that both helper T cells and various histiocytes, including FDC, express the Leu-3/T4 antigen[5,6] which is an essential component of the cell surface receptor necessary for HTLV-III/LAV infection.[7] Furthermore, it has been shown previously that certain types of histiocytes can be infected by HTLV-III/LAV.[7] Interestingly, Parravicini et al were unable to detect reactivity of other lymph node cells, including helper T cells, with their antibody. This may be related to the fact that less than 0.01% of peripheral blood or lymph node mononuclear cells from individuals infected with HTLV-III contain HLTV-III RNA detectable by an in situ hybridization technique.[8] It is possible that among other Leu-3+/T4+ cells, such as FDC, a larger proportion of cells may contain detectable HTLV-III/LAV. Of course, it will be important to distinguish between active retroviral infection of FDC and their passive acquisition of retroviral antigens, eg, by adsorption or phagocytosis.

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MIXED LINEAGE LEUKEMIA: THE IMPLICATIONS FOR HEMATOPOIETIC DIFFERENTIATION

To the Editor:

Greaves and colleagues[1] recently reviewed the evidence for the violation of lineage fidelity in the human leukemias. They propose a model suggesting that "apparent cases of lineage infidelity do not reflect genetic misprogramming but rather a transient phase of limited promiscuity of gene expression occurring in normal bipotential or multipotential progenitors and able to be preserved as a relic in leukemic blast cell populations that are in maturation arrest." While acknowledging that "aberrant gene expression may well occur in leukemia," the authors conclude that such cases occur "with insufficient regularity to be of general significance."

The simultaneous expression of genes encoding for myeloid and lymphoid differentiation demonstrated by the reactivity of monoclonal antibodies (MoAbs) with surface antigens might be criticized as antibody recognition of a cross-reacting epitope, or as a MoAb identifying an antigen that is widely expressed and not restricted to a single lineage. However, we have eliminated the possibility of a cross-reacting epitope for the E-rosette–associated antigen (Tp50 molecule) by immunoprecipitating this molecule from myeloblasts.[2] Nor can the possible difficulty of widely expressed antigens explain our detection of as many as four myeloid-associated antigens on blasts from patients with acute lymphoblastic leukemia (ALL).[3] Moreover, some cell surface antigens such as T3 normally appear late in differentiation and their presence on myeloblasts is difficult to explain on the basis of mosaic expression of genes during early differentiation.

In an attempt to determine the frequency and clinical significance of acute leukemia displaying both lymphoid and myeloid characteristics we undertook a prospective study using a large panel of lineage-associated markers which included DNA analysis.[3] We identified myeloid-associated features in 19% of 95 consecutive cases of otherwise typical ALL and lymphoid-associated features in 25% of 28 consecutive cases that met standard criteria for acute myeloid leukemia (AML).[2,3] Dual staining techniques including microsphere-conjugated monoclonal antibodies and flow cytometric analysis demonstrated that individual blast cells were simultaneously expressing lymphoid- and myeloid-associated antigens, with some cases simultaneously expressing T cell, B cell, and myeloid characteristics. We have termed such cases "acute mixed-lineage leukemia."[3,4] We have now studied a total of 260 consecutive children with acute leukemia, and our early results documenting the frequency and spectrum of mixed lineage expression are being confirmed.[5] Furthermore, we have extended our findings to the DNA level and have documented mixed gene expression in typical cases of acute leukemia[6,7] and in cases of mixed lineage leukemia.[3,8]

Earlier studies by McCulloch and colleagues indicated that acute leukemia with mixed-lineage characteristics confers a poor prognosis.[9] We believe the clinical importance of acute mixed lineage leukemia is now beginning to emerge. For patients with otherwise typical morphologic features of ALL, the expression of specific myeloid-associated surface antigens correlates with a poor response to induction therapy.[10] Furthermore, we have treated four patients with AML whose blasts expressed the T11 antigen (Tp50 molecule) that failed myeloid-directed therapy and then responded dramatically to treatment generally used for ALL.

We concur with Greaves that pluri- or bipotent progenitor cells may be able to coexpress lymphoid and myeloid differentiation-linked genes before they commit to a particular lineage, and have suggested that leukemic transformation of such cells offers an explanation for cases of mixed-lineage leukemia.[2,3,11] In fact, mosaic expression of differentiation-associated surface antigens which function as receptors (eg, T11) may provide the means for external factors to affect lineage commitment. However, evidence suggests that other mechanisms may contribute to the high frequency of anomalous phenotypes and genotypes that we and others have identified in acute leukemias. One of these mechanisms may involve rearrangements of DNA (ie, reciprocal translocations, inversions, or partial deletions) that potentially could disrupt the sequences that normally control transcription of genes encoding specific differentiation antigens. Yunis, for example, found chromosomal abnormalities in virtually all cases of AML he examined,[12] while Williams et al recently reported such changes in 94% of 116 cases of ALL.[13] In our patients expressing mixed lineage characteristics we commonly identified complex cytogenetic abnormalities. Thus an almost infinite variety of cell lineage–related phenotypes and genotypes might result from this mechanism, whether or not a pluripotent stem cell was the target of leukemic transformation.

This model of aberrant differentiation predicts that a high proportion of cases with specific chromosomal translocations that disrupt genetic control would be expected to show features of mixed-lineage leukemia. In a relatively large series of cases with the 4;11 translocation we found mixed-lineage characteristics whenever the blast cells could be extensively analyzed.[14] Individual blasts coexpressed myeloid- and lymphoid-associated surface antigens as well as ultrastructural characteristics of both lineages. Others have found that cases with 11q23 abnormalities express mixed phenotypic characteristics.[15] This information combined with our data from the t(4;11)(q21;q23) cases suggests that the q23 breakpoint on chromosome 11 may be important in the simultaneous expression of myeloid and lymphoid characteristics. Further identification of cases with specific chromosomal abnormalities other than 11q23 that are associated with mixed lineage leukemia would provide further support for a model based on the loss of genetic control.

Collectively, our findings indicate that unusual combinations of myeloid and lymphoid cell lineages are more common in acute leukemia than has been generally recognized or suspected, and that more than one mechanism may be responsible for their genesis. We propose the general term "mixed-lineage leukemia" in place of "biphenotypic leukemia" (implying two populations of cells), "hybrid phenotype" (suggesting a fusion of elements), or "lineage infidelity" (which suggests aberrant gene expression). Extension of mixed-lineage characteristics to the genomic level in a number of articles[3,6-8] limits the accuracy of earlier terms such as "mixed lymphoid-myeloid phenotype"[16] or "polyphenotypic leukemia." Since lineage promiscuity makes no provision for genetic misprogramming, we feel it adds an unnecessary term to an already complex literature and would object to its application to cases such as ours. Because we believe both mechanisms, genetic misprogramming and mosaic gene expression, may be responsible for the genesis of mixed-lineage leukemia, molecular studies of such cases might provide important information about hematopoietic differentiation.

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To the Editor:

Our review article[1] was written to provoke debate and we welcome the response from Mirro and colleagues,[2] particularly because they have contributed much of the primary data relevant to the subject under discussion.

There are three distinct issues which are somewhat intertwined in the letter from Mirro et al; we feel it is important to consider them separately. First, Mirro et al prefer the term "mixed lineage" leukemia to describe acute leukemias in which markers of more than one lineage are expressed on individual blast cells, and they disapprove of our term "promiscuity"[1] in this context. Of course, we never suggested in all seriousness that such leukemias should be labeled as "promiscuous" acute leukemias; this was, as most will realize, a tongue-in-cheek term coined in response to the infidelity argument[3,4] and was used to help encapsulate a potential biological explanation for mixed phenotypes. We currently refer to such leukemias operationally as "unclassifiable" and use the term "mixed lineage" for those cases in which two or more leukemic cell populations belonging to different lineages coexist.

Second, Mirro and colleagues emphasize that "mixed lineage" leukemias may have an inferior prognosis as suggested earlier by Smith et al.[4] From the data presented this seems likely to be correct for those cases diagnosed as AML but not (overall) for ALL with "myeloid" markers.[4,5] We acknowledged in our review[1] that this association might well exist but emphasized that it has no bearing on the issue of whether "mixed lineage"gene expression reflects aberrant or normal differentiation.

Third, Mirro et al conclude, as we did, that multiple mechanisms may underlie the expression of apparently "mixed lineage" phenotypes in acute leukemia but prefer to believe that genetic misprogramming, as suggested earlier by McCulloch and colleagues,[3,4] perhaps resulting from chromosomal rearrangements, is a major underlying cause. The supporting data they provide are compatible with this idea but have questionable credentials as prima facie evidence. Because only a small proportion of acute leukemias have apparently "mixed lineage" phenotypes and as yet these are not found associated with particular karyotypic abnormalities, the common occurrence of a wide variety of chromosome changes to which Mirro et al refer[2] should not be construed as evidence.

Mirro et al[2] also restate their empirical observations on "mixed lineage" phenotypes[5,6] and suggest that these are difficult to interpret, as we suggested, by a mosaic pattern of gene expression or inherent flexibility of lineage options in early differentiation. Without wishing to reiterate much of our review[1] we must respectfully challenge Mirro and his associates on this point because they ignore the crucial issue of whether the antimyeloid monoclonal antibodies used are really myeloid specific. They state that up to four myeloid associated antigens may be expressed on ALL cells. In fact, only two cases out of 95 ALL investigated expressed four such antigens (cases 3 and 4 in Table 1, ref 5). More importantly, the antibodies used (MCS.2/CDw13, Mo1/CD11, SJ-D1, and 5F1/CDw14, and excluding antihapten X antibodies MSC.1 and My-1) have not, as far as we are aware, been assessed for reactivity with TdT positive lymphoid cells in normal or regenerating bone marrow. The myeloid restriction of these reagents' reactivity is a presumption, not a fact. Because antibody SJ-D1 on which Mirro et al place some emphasis[5] has been reported to react with endothelia, epithelia, kidney tubules, and normal thymocytes[7], the alarm bells should be ringing.

Mirro et al[2] also suggest that even a "late" lymphoid differentiation antigen such as T3 (CD3) can be expressed in AML providing contrary evidence to our suggestion that lineage promiscuity might be limited to "early" antigens. We can only find three AML patients with this feature in the reports of Mirro et al; two of these appear to be the same patient (No. 19 in ref 5, No. 1 in ref 6). The myeloid reactivity of these patients was, in fact, unconvincing. Indeed, because these cells had lymphoid morphology, several lymphoid markers, less than 15% positivity for myeloid enzymes, and reactivity with only one putative antimyeloid antibody (Mo1) and yet were classified as ANLL,[4,5] it is evident that strict adherence to the FAB classification scheme[8] is a confusing factor in these studies. Furthermore, recent studies indicate that T3 proteins (γ, δ, ϵ) are very early T lineage–associated gene products that only transport to the cell surface in the presence of the heterodimeric α/β T cell receptor proteins.[9] It would therefore be quite extraordinary if myeloid leukemic cells succeeded in a full, functional activation of this complete set of T cell receptor genes. Along the same lines, Mirro et al reiterate the now common finding that rearrangements of Ig and T cell receptor genes occur in leukemic cells belonging to the "inappropriate" lymphoid lineage (and more rarely in AML). We emphasized in our article that such rearrangements are partial, nonfunctional and a component of normal differentiation. Yancopoulos et al have now demonstrated that both receptor gene loci can be rearranged by the same "recombinase" activity in individual cells.[10]

We agree that "mixed lineage" acute leukemias (or whatever one wishes to call them) are more common than appreciated earlier and that they constitute a biologically and clinically important subgroup. Whether the underlying molecular mechanisms responsible for these odd or unanticipated phenotypes are a reflection of normal differentiation programming, leukemia-associated events, or both, remains to be established.

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ANNOUNCEMENTS

RECENT ADVANCES IN LEUKEMIA AND LYMPHOMA

Organizers: Robert Peter Gale and David W. Golde

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Abstract Submission Deadlines: *, October 3, 1986; †, November 26, 1986. Applications will be accepted at any time for meetings which are not over-subscribed. *For further information,* please call or write: UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, CA 90024. Telephone: (213) 206-6292. Telex: UCLA Symposia 9103427597.

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The Twenty-Eighth Annual Meeting of the American Society of Hematology will be held at the Moscone Center, San Francisco, Dec 6–9, 1986. Programs by the Education Committee and the Scientific Subcommittees will be held on Saturday, Dec 6, and Sunday, Dec 7. The Plenary Session will be held Sunday afternoon, Dec 7, and the Presidential Symposium will be Tuesday morning, Dec 9. Selected papers submitted to the meeting will be presented either in oral or poster sessions on Monday, Dec 8, and Tuesday, Dec 9.

The abstract deadline for contributed papers is Sept 5, 1986. Abstract forms may be obtained from SLACK, Inc. (address below). Subsequent issues of *Blood* will contain details of the Education and Subcommittee Programs and the Presidential Symposium, in addition to information about registration to the meeting. Preregistration material will be sent early this fall to members. If you do not receive your meeting information by Oct 6, 1986, please contact the Registration Supervisor at SLACK, Inc, Box 510, Thorofare, NJ 08086-0510, (609)848-1000 for this important information. Nonmembers wishing to receive preregistration information and forms should also contact SLACK, Inc.

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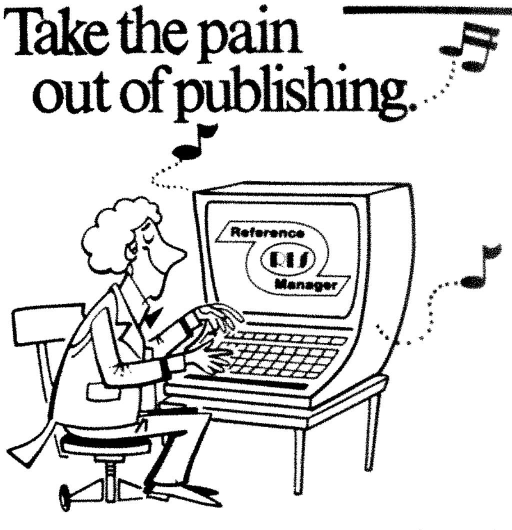

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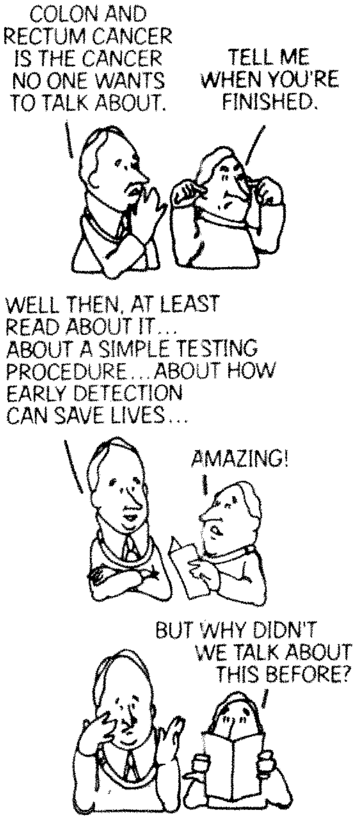
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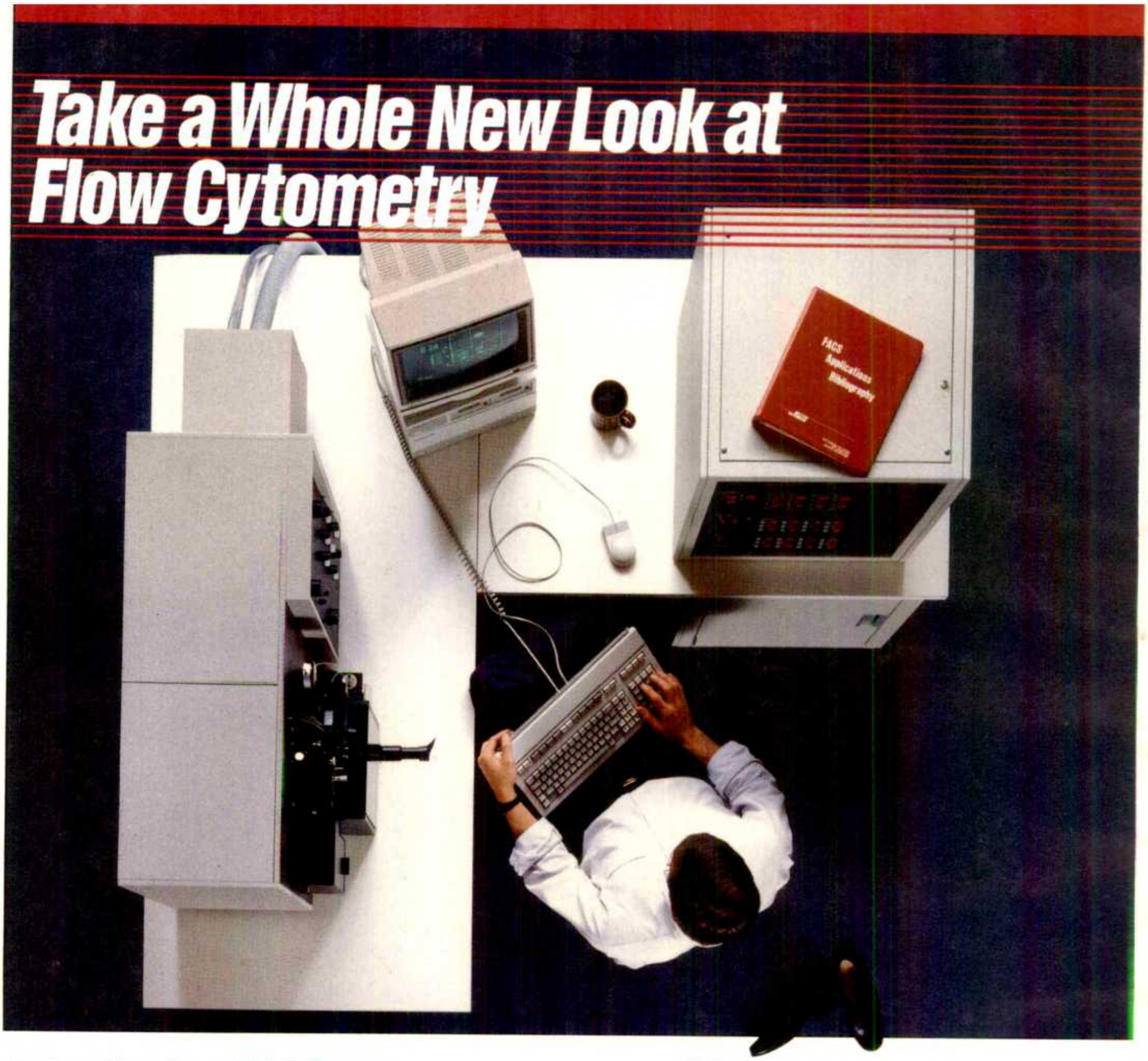
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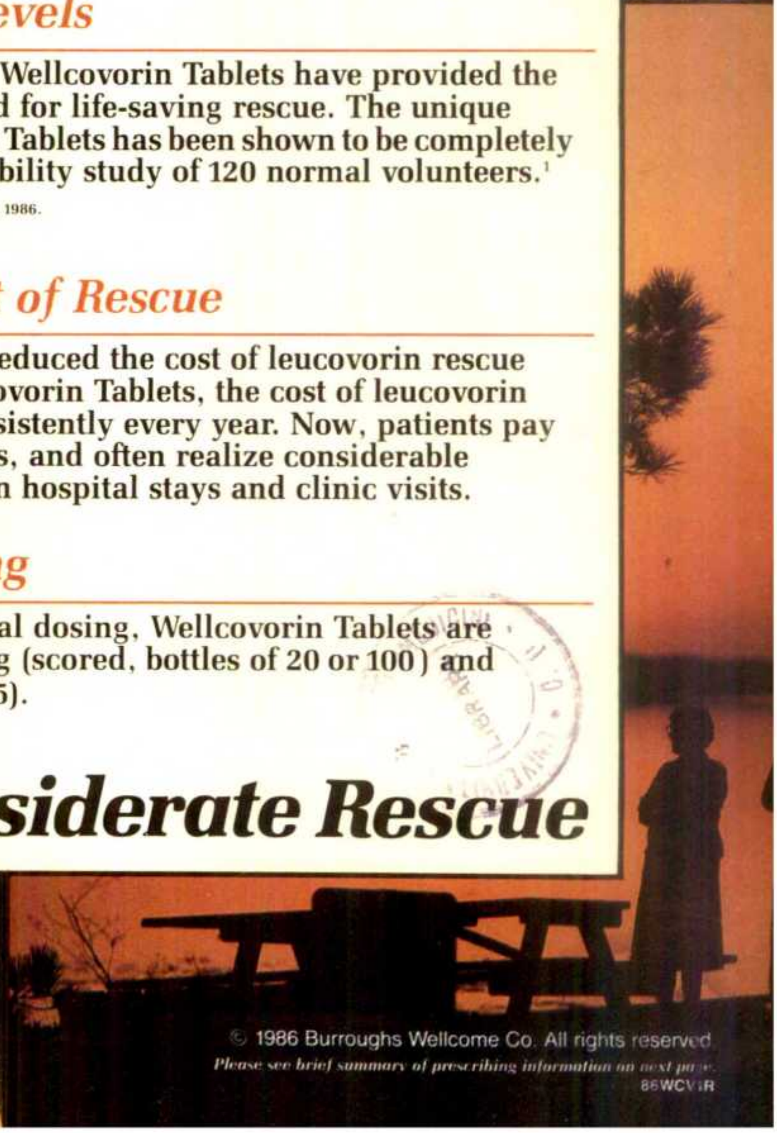

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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

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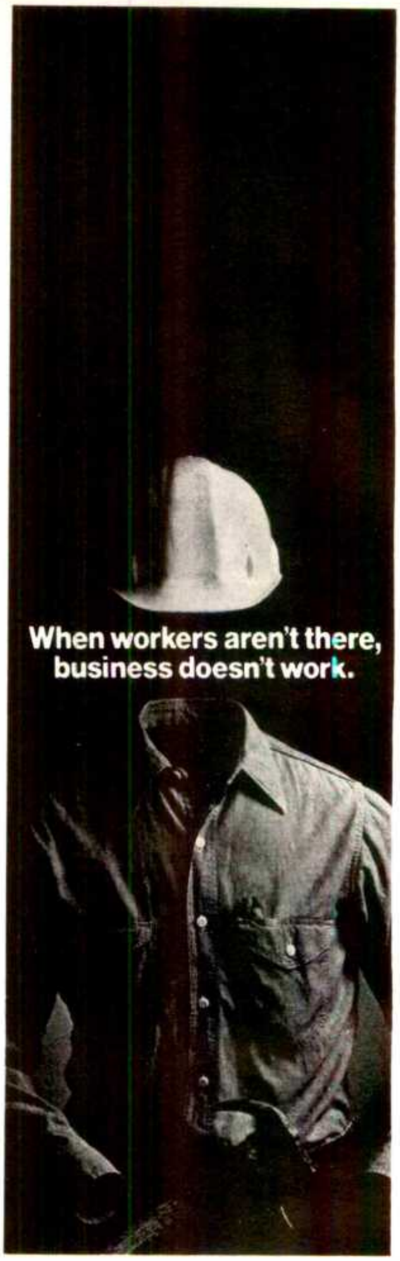

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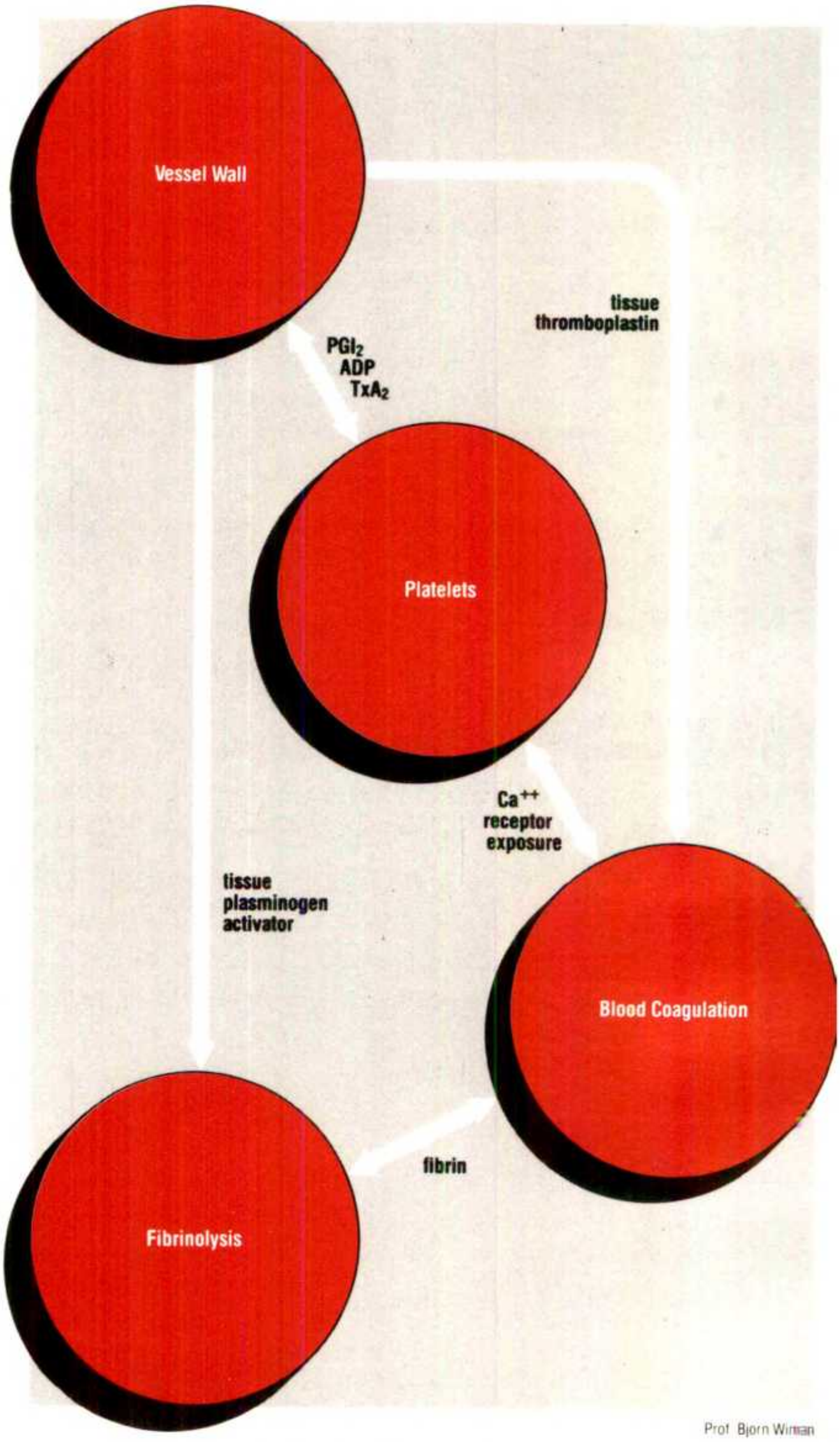

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RED CELL METABOLISM

A MANUAL OF BIOCHEMICAL METHODS
Third Edition
ERNEST BEUTLER, M.D.

This third edition of **Red Cell Metabolism: A Manual of Biochemical Methods** presents a detailed discussion of the methods required for the measurement of red cell enzymes and metabolic intermediates. The techniques described—which have evolved after years of performing these assays in a laboratory environment—are essential both to the clinical diagnosis of certain types of anemia and to basic research on red blood cells. Topics covered include the fundamentals of handling blood samples, methods of reagent preparation, instrumentation, and calculation of results. New methods, improved procedures, and the increasing importance of certain red cell constituents such as GSH-S-transferase, superoxide dismutase, Na^{+}-K^{+}-ATPase, phosphoglycolate phosphatase, and creatine are among the factors that have brought about the need for this updated edition of the text.

Written for hematologists, clinical pathologists, biochemists, and cellular biologists, the third edition of **Red Cell Metabolism** represents the most authoritative and current information in the field of red cell enzymology, and is internationally recognized as the classic text on the subject.

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Introduction: Purpose and Organization of the Manual. Red Cell Metabolism. **Basic Techniques and Equipment:** The Preparation of Red Cells for Assay. Reagents. Instrumentation. Calculation of Results. **Red Cell Glycolytic Enzymes:** Hexokinase (Hx). Glucose Phosphate Isomerase (GPI). Phosphofructokinase (PFK). Aldolase. Triose Phosphate Isomerase (TPI). Glyceraldehyde Phosphate Dehydrogenase (GAPD). Phosphoglycerate Kinase (PGK). Diphosphoglycerate Mutase (DPGM). Monophosphoglyceromutase (MPGM). Enolase. Pyruvate Kinase (PK). Lactate Dehydrogenase (LDH). **Red Cell Hexose Monophosphate Shunt and Other Enzymes:** Glucose-6-Phosphate Dehydrogenase (G-6-PD) and 6-Phosphogluconate Dehydrogenase (6-PGD). Glutathione Reductase (GR). Glutathione Peroxidase (GSH-Px). Glutathione S-Transferase. NADPH Diaphorase. NADH Methemoglobin Reductase (NADH-Ferricyanide Reductase). Superoxide Dismutase. Phosphoglucomutase (PGM). Glutamate-Oxaloacetate Transaminase (GOT). Phosphoglycolate Phosphatase. Adenylate Kinase (AK). Na^{+}-K^{+}ATPase and Mg^{+} $^{+}$ATPase. Adenosine Demmainase (AD). Pyrimidine 5′ Nucleotidase. Acetylcholinesterase. Catalase. Galactose-1-Phosphate Uridyl Transferase. Galactokinase (GK). UDP Glucose-4-Epimerase (Epimerase). **Intermediate Compounds:** ATP. AMP and ADP. 2,3-Diphosphoglycerate (2,3-DPG). Reduced Glutathione (GSH). Oxidized Glutathione (GSSG). Creatine. Glucose. Pyruvate. Lactate. Glucose-6-P and Fructose-6-P. Glyceraldehyde-3-P (GAP), Dihydroxyacetone-P (DHAP), and Fructose-DiP (FDP). 3-Phosphoglyceric Acid (3-GPA). 2-Phosphoglyceric Acid (2-PGA) and Phosphoenolpyruvate. **Screening Techniques:** General Principles. Glucose Phosphate Isomerase Screening Test. Triose Phosphate Isomerase Screening Test. Pyruvate Kinase Screening Test. Glucose-6-Phosphate Dehydrogenase Screening Test. Glutathione Reductase Screening Test. NADH Methemoglobin Reductase (NADH Diaphorase). Pyrimidine 5′ Nucleotidase Screening Test. **Appendix 1:** Activities of Red Cell Enzymes in Normal Adults. **Appendix 2:** Effect of Temperature on Red Cell Enzyme Assays. **Appendix 3:** Levels of Intermediates in Red Cells of Normal Adults. **Appendix 4:** Frequently Used Molecular Weights.

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irst recombinant nterferon into the clinic

ith more than a decade of dedicated esearch, Roche leads the way in iden- fying, purifying and engineering the rst recombinant alfa Interferon

1969, scientists at the Roche Institute of olecular Biology began the intensive ork that culminated in the production of fa Interferon in *E. coli* cells by means of ene-splicing and cloning technology. An dvanced technique of affinity chromatog- aphy using monoclonal antibodies assures virtually pure product.

ulticenter study included leading cancer centers...large atient data base

1981, ROFERON-A became the first ecombinant Interferon used in clinical ials at six leading cancer centers through- ut the United States. The remarkable clini- al response achieved in these trials stablished ROFERON-A as breakthrough iotherapy for hairy cell leukemia.

Study utilized fixed-dosage SC or IM injectable solution to permit self-injection by patient or administration by family member in the home

Home administration reduces the demands on busy medical staff and spares the patient time, travel "wear and tear" and expense of making frequent office or clinic visits.

Fixed-dosage SC or IM injectable solution allows administration without calculation in home, office or institution...saves time, cost-effective

Elimination of weight or body surface area calculation results in more efficient administration with less waste of time and medication. Available as ready-to-use injectable solution with no mixing or reconstitution necessary.

Common side effects usually self-limiting and manageable

Initial side effects, such as fever, chills, myalgia, headache and fatigue, are self-limiting and generally manageable. Myelosuppression, which can occur early in the treatment course, warrants close clinical and laboratory observation and is usually alleviated by dose reduction or temporary discontinuation of drug.

EW SC/IM

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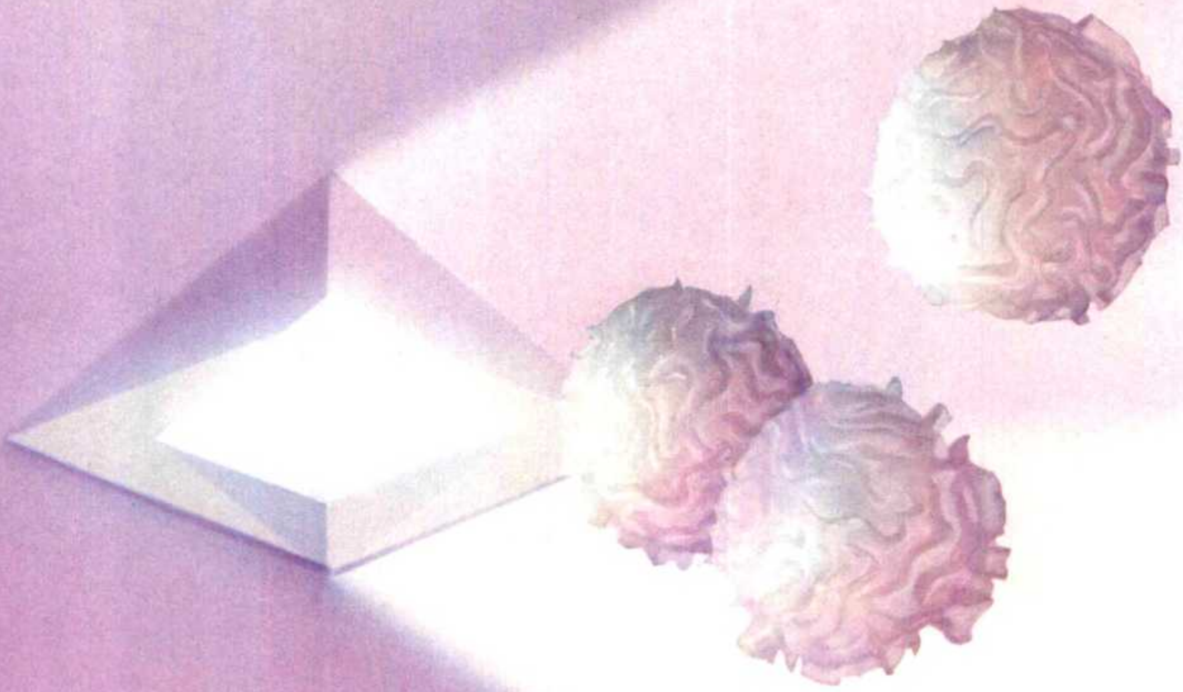
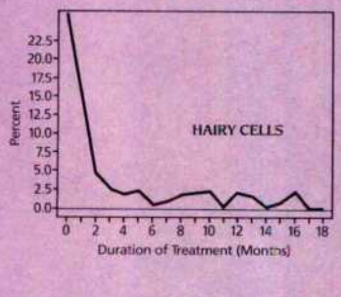
In hairy cell leukemia

The clinical response





Artist's interpretation of hairy cells based on scanning electron microscopy

With normalization of all peripheral blood counts, concomitant decrease in peripheral blood hairy cells*

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is remarkable

Statistically increased survival compared to a historical control group 89% improved, 11% stabilized

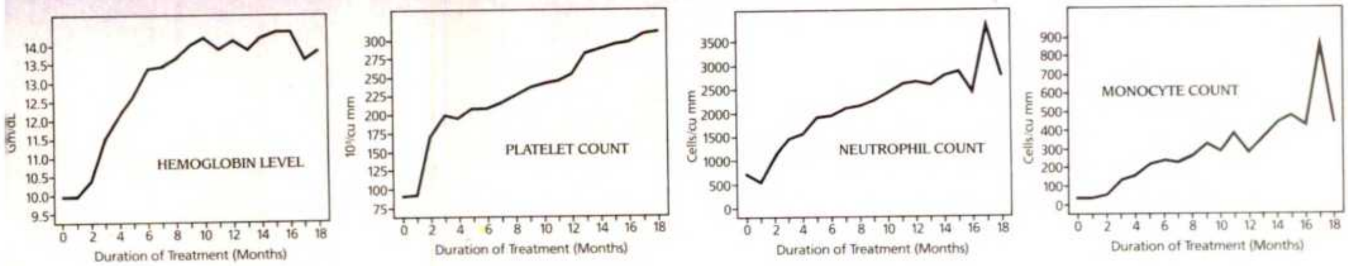
Treatment of hairy cell leukemia: results of multicenter study*

No. of patients evaluable for efficacy	Dosage (IU = International Units)	Response			Median Time to Response (Days)		
		Complete or Partial Remission	Minor Remission	Stabilized	Complete Remission	Partial Remission	Minor Remission
75[†]	Induction: 3 million IU SC/IM once-daily for 6 months Maintenance: 3 million IU SC/IM 3 times/week	46 **(61%)**	21 **(28%)**	8 **(11%)**	249	133	57

Response Criteria

	Complete	Partial	Minor
Bone Marrow Hairy Cells	0	> 50% decrease of HCL infiltrate from pretreatment values	< 50% decrease of HCL infiltrate from pretreatment values
Bone Marrow Granulocytes (sum of % of Myelocytes, Metamyelocytes and Neutrophils)	> 35%	> 35%	25-35%
Hemoglobin	> 12 Gm/dL	> 12 Gm/dL	Improvement
Absolute Neutrophil Count	> 1500 cells/cu mm	> 1500 cells/cu mm	Improvement
Platelet Count	> 100,000/cu mm	> 100,000/cu mm	Improvement

Normalization of blood counts*



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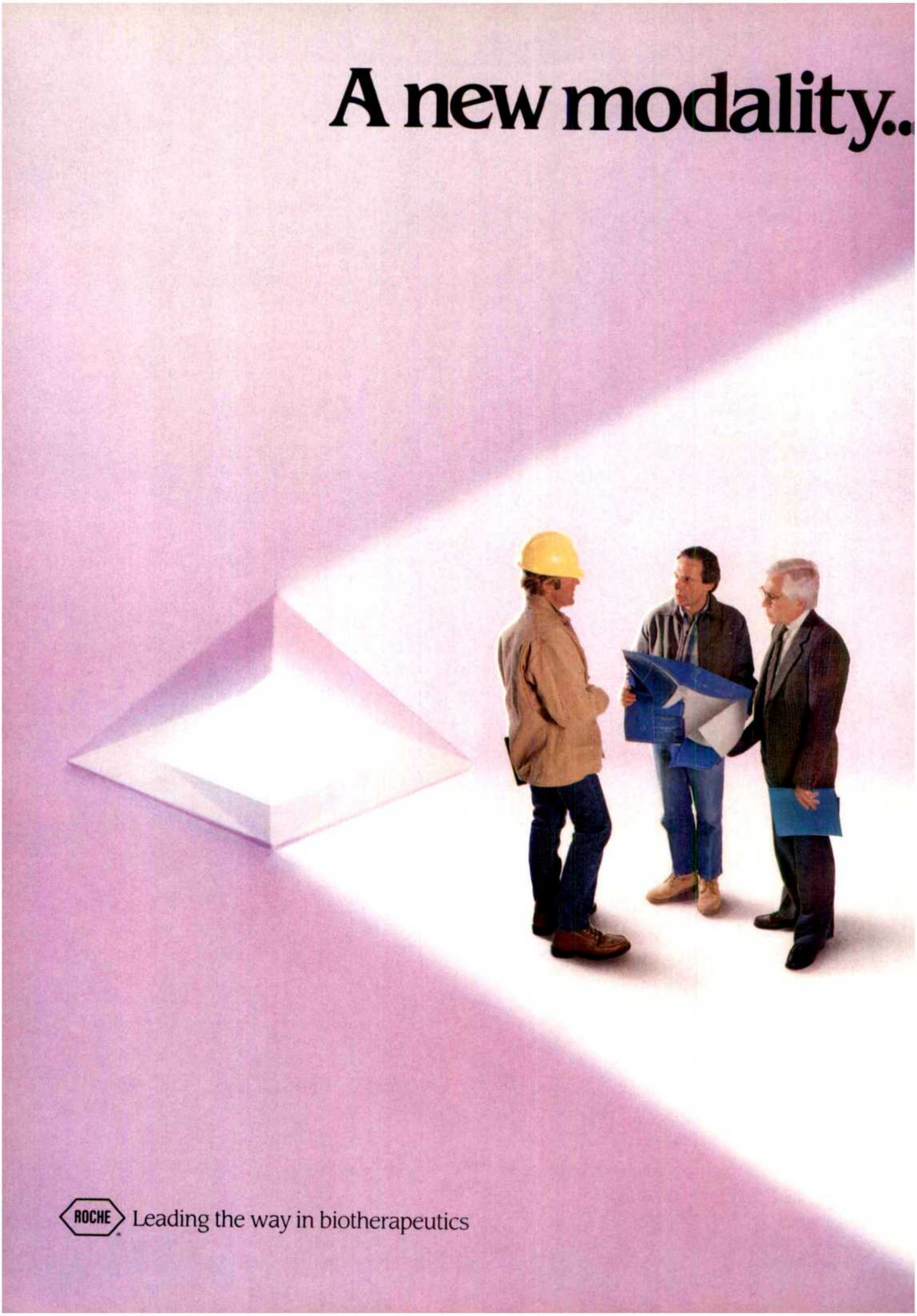
[*] Multicenter Study. Data on ile, Hoffmann-La Roche nc., Nutley, NJ.

[†] Of the 75, 57 had prior splenectomy and 18 had not; many were pancytopenic and transfusion-dependent; 96 patients were enrolled in study and all were evaluated for safety; 21 patients were not evaluable; 16 were not on drug long enough for efficacy evaluation at time of data analysis, 2 were removed for administrative reasons, 3 for preexisting intercurrent illness.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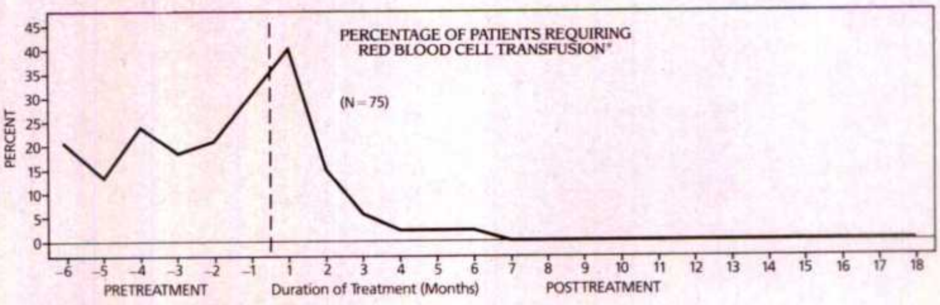
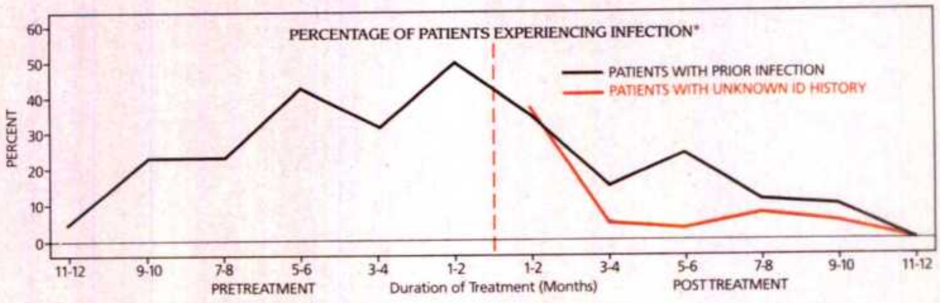
a better quality of life

From transfusion dependency to independent ambulatory treatment



From frequent life-threatening pportunistic infections to virtually none



*Multicenter Study, Data on file, Hoffmann-La Roche Inc., Nutley, NJ.

From frequent hospital visits to self-injection at home

After an initial period of hospital or clinic visits for drug administration, self-injection at home or administration by a family member makes it possible for many patients to lead a more normal life.

From the serious, difficult-to-manage side effects of chemotherapy to the more manageable side effects of biotherapy

The common side effects of ROFERON-A are rarely as debilitating as some of the serious side effects associated with standard chemotherapy and radiotherapy. (See side effects listed on following pages and in complete product information on last pages of this advertisement.)

From weight loss to weight stabilization

In a multicenter study, 86% of patients on ROFERON-A experienced no change in, or gained up to 10% of, their total body weight.

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Interferon BRAND OF alfa-2a, recombinant/Roche

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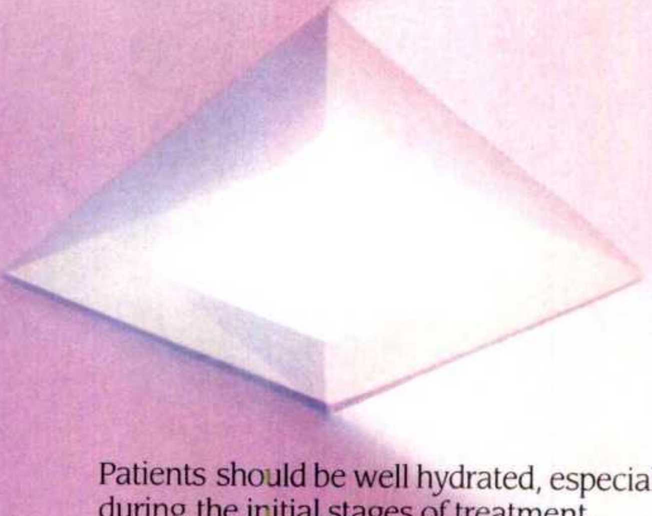
Guidelines to treatmen

Patient selection

ROFERON-A (Interferon alfa-2a, recombinant/Roche) is indicated for symptomatic adult patients. It should be used with caution in patients with preexisting cardiac disease, renal and hepatic disease, myelosuppression, seizure disorders and/or compromised central nervous system function.

Information for patients

Patients should be cautioned not to change brands of Interferon without medical consultation, as a change in dosage may result. They should also be informed regarding the potential benefits and risks attendant to the use of ROFERON-A. If home use is judged desirable, instructions on appropriate use and self-injection should be given.



Patients should be well hydrated, especially during the initial stages of treatment.

Dosage and administration

Induction:	**3 million IU SC or IM once daily for 6 months.**
Maintenance:	**3 million IU SC or IM 3 times a week.**
Dosage Reduction:	**If severe reactions occur, reduce dosage by 50% or withhold individual doses.**

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How supplied

Ready-to-use Injectable Solution: Unit-dos vials containing 3 million IU (3 million IU/mL); multidose vials containing 18 million IU (3 million IU/0.5 mL).
No mixing or reconstitution necessary.
Must be refrigerated at temperatures of 36° to 46°F (2° to 8°C). At no time should ROFERON-A (Interferon alfa-2a, recombinant/Roche) Injectable Solution remain outside of refrigeration for more than 24 hours. Be certain all users, including physicians, nurses and patients are aware of thi important precaution. Do *not* freeze or sha

Directions for use

The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are significantly thrombocytopenic or at risk for bleeding.

Side effects: generally mild and manageable

Pretreatment

Routine history and physical examination. Determine baseline values: CBC, peripheral and bone marrow hairy cells, liver and renal function.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Flu-like symptoms

The most common initial reactions, fever, chills, myalgia, headache and fatigue, are usually self-limiting and can be ameliorated by acetaminophen.

Myelosuppressive effect

The myelosuppressive effect warrants careful monitoring for depression of blood cell

counts during initial therapy and is usually alleviated by dose reduction or temporary discontinuation of drug.

Other common but generally mild side effects

These include anorexia, nausea, vomiting, diarrhea, dizziness, rash, change in taste, dry mouth or throat, dry skin or pruritus and weight loss. Persistent mild fatigue, a problem for many patients, is usually found to be less troublesome when ROFERON-A is administered *p.m.* or *h.s.*

Rarely reported but more serious side effects

These include, but are not limited to, central nervous system effects such as decreased mental status, depression, visual disturbances, sleep disturbances and nervousness, as well as such adverse cardiac events as hypertension, chest pain, arrhythmias and palpitations.

Patient monitoring

Monitor blood parameters, liver and renal function periodically (*e.g.*, monthly) to determine if patient is responding to and tolerating drug. Response is not generally observed for one to three months. Patients usually should be treated for approximately six months before determining whether to continue therapy.

Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.

Laboratory findings

No patient had to be removed from multicenter study due to clinical laboratory abnormality.
Laboratory abnormalities, observed frequently but generally not of clinical significance, include:

SGOT elevation
  Common but not dose-limiting

Neutralizing antibodies
  Found in four out of 51 (7.8%) patients in multicenter study evaluable for the development of antibodies; no clinical sequelae or adverse effect on response, duration of response or survival has been noted thus far.

NOTE: Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.

Contraindications

ROFERON-A is contraindicated in patients with known hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

Use in fertile women

Fertile women should use effective contraception while receiving ROFERON-A.

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BRAND OF
Interferon alfa-2a, recombinant/Roche

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ROFERON®-A

Interferon (BRAND OF) alfa-2a, recombinant/Roche

DESCRIPTION: Roferon®-A (Interferon alfa-2a, recombinant/Roche) is a sterile protein product for use by injection. Roferon-A is manufactured by recombinant DNA technology that employs a genetically engineered *E. coli* bacterium containing DNA that codes for the human protein. Interferon alfa-2a, recombinant/Roche is a highly purified protein containing 165 amino acids, and it has an approximate molecular weight of 19,000 daltons. The purification procedure includes affinity chromatography using a murine monoclonal antibody. Roferon-A is supplied as an injectable solution. The solution is colorless and contains Interferon alfa-2a, recombinant/Roche, phenol as a preservative, sodium chloride for isotonicity and human serum albumin as a stabilizer. Fermentation is carried out in a defined nutrient medium containing the antibiotic tetracycline hydrochloride, 5 mg/liter. However, the presence of the antibiotic is not detectable in the final product.

Each vial of Roferon-A contains 3 million or 18 million international units (IU) of Interferon alfa-2a, recombinant/Roche. The specific activity of Interferon alfa-2a, recombinant/Roche, is 2×10^8 IU/mg protein. The route of administration is subcutaneous or intramuscular.

CLINICAL PHARMACOLOGY: The mechanism by which Interferon alfa-2a, recombinant/Roche, or any other interferon, exerts antitumor activity is not clearly understood. However, it is believed that direct antiproliferative action against tumor cells and modulation of the host immune response play important roles in the antitumor activity.

The biological activities of Interferon alfa-2a, recombinant/Roche are species-restricted, *i.e.*, they are expressed in a very limited number of species other than humans. As a consequence, preclinical evaluation of Interferon alfa-2a, recombinant/Roche has involved *in vitro* experiments with human cells and some *in vivo* experiments.[1] Using human cells in culture, Interferon alfa-2a, recombinant/Roche has been shown to have antiproliferative and immunomodulatory activities that are very similar to those of the mixture of interferon alfa subtypes produced by human leukocytes. *In vivo*, Interferon alfa-2a, recombinant/Roche has been shown to inhibit the growth of several human tumors growing in immunocompromised (nude) mice. Because of its species-restricted activity, it has not been possible to demonstrate antitumor activity in immunologically intact syngeneic tumor model systems, where effects on the host immune system would be observable. However, such antitumor activity has been repeatedly demonstrated with, for example, mouse interferon-alfa in transplantable mouse tumor systems. The clinical significance of these findings is unknown.

The metabolism of Interferon alfa-2a, recombinant/Roche is consistent with that of alfa interferons in general. Alfa interferons are totally filtered through the glomeruli and undergo rapid proteolytic degradation during tubular reabsorption, rendering a negligible reappearance of intact alfa interferon in the systemic circulation. Small amounts of radiolabeled Interferon alfa-2a, recombinant/Roche appear in the urine of isolated rat kidneys, suggesting near complete reabsorption of Interferon alfa-2a, recombinant/Roche catabolites. Liver metabolism and subsequent biliary excretion are considered minor pathways of elimination for alfa interferons.

The serum concentrations of Interferon alfa-2a, recombinant/Roche reflected a large intersubject variation in both healthy volunteers and patients with disseminated cancer.

In healthy people, Interferon alfa-2a, recombinant/Roche exhibited an elimination half-life of 3.7 to 8.5 hours (mean 5.1 hours), volume of distribution at steady-state of 0.223 to 0.748 L/kg (mean 0.400 L/kg) and a total body clearance of 2.14 to 3.62 mL/min/kg (mean 2.79 mL/min/kg) after a 36 million IU (2.2×10^8 pg) intravenous infusion. After intramuscular and subcutaneous administrations of 36 million IU, peak serum concentrations ranged from 1500 to 2580 pg/mL (mean 2020 pg/mL) at a mean time to peak of 3.8 hours and from 1250 to 2320 pg/mL (mean 1730 pg/mL) at a mean time to peak of 7.3 hours, respectively. The apparent fraction of the dose absorbed after intramuscular injection was greater than 80%.

The pharmacokinetics of Interferon alfa-2a, recombinant/Roche after single intramuscular doses to patients with disseminated cancer were similar to those found in healthy volunteers. Dose proportional increases in serum concentrations were observed after single doses up to 198 million IU. There were no changes in the distribution or elimination of Interferon alfa-2a, recombinant/Roche during twice daily (0.5 to 36 million IU), once daily (1 to 54 million IU), or three times weekly (1 to 136 million IU) dosing regimens up to 28 days of dosing. Multiple intramuscular doses of Interferon alfa-2a, recombinant/Roche resulted in an accumulation of 2 to 4 times the single dose serum concentrations. Pharmacokinetic information in patients with hairy cell leukemia is presently unknown.

The acute parenteral toxicity of Interferon alfa-2a, recombinant/Roche has been studied in mice, rats, rabbits and ferrets at doses up to 30 million IU/kg intravenously, and 500 million IU/kg intramuscularly. No treatment-related mortality was noted in any species given Interferon alfa-2a, recombinant/Roche by any of the routes of administration.

Effects on Hairy Cell Leukemia: During the first one to two months of treatment of patients with hairy cell leukemia, significant depression of hematopoiesis was likely to occur. Subsequently, there was improvement in circulating blood cell counts.

Of the 75 patients who were evaluable for efficacy following at least 16 weeks of therapy, 46 (61%) achieved complete or partial response. Twenty-one patients (28%) had a minor remission, eight (11%) remained stable, and none had worsening of disease. All patients who achieved either a complete or partial response had complete or partial normalization of all peripheral blood elements including hemoglobin level, white blood cell, neutrophil, monocyte and platelet counts with a concomitant decrease in peripheral blood and bone marrow hairy cells. Responding patients also exhibited a marked reduction in red blood cell and platelet transfusion requirements, a decrease in infectious episodes and improvement in performance status. The probability of survival for two years in patients receiving Roferon-A (94%) was statistically increased compared to a historical control group (75%).

ROFERON®-A (Interferon alfa-2a, recombinant/Roche)

INDICATIONS AND USAGE: Roferon-A is indicated for use in the treatmen of hairy cell leukemia in people 18 years of age or older. Studies have shown that Roferon-A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and nonsplenectomized patients.[2,3]

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodicall (*e.g.*, monthly) during treatment to determine whether response to treatmen has occurred. If a patient does not respond within six months, treatment should be discontinued. If a response to treatment does occur, treatment should be continued until no further improvement is observed and these laboratory parameters have been stable for about three months. It is not known whether continued treatment after that time is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS: Roferon-A is contraindicated in patients with known hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

WARNINGS: Roferon-A should be administered under the guidance of a qualified physician. (See DOSAGE AND ADMINISTRATION.) Appropriate management of the therapy and its complications is possible only when adequate diagnostic and treatment facilities are readily available.

Roferon-A should be used with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised central nervous system function.

Because of the possibility of severe or even fatal adverse reactions, patients should be informed not only of the benefits of therapy but also of the risks involved.

Roferon-A should be administered with caution to patients with cardiac disease or with any history of cardiac illness. No direct cardiotoxic effect has been demonstrated, but it is likely that acute, self-limited toxicities (*i.e.*, feve chills) frequently associated with Roferon-A administration may exacerbate preexisting cardiac conditions. Rarely, myocardial infarction has occurred in patients receiving Roferon-A.

Caution should be exercised when administering Roferon-A to patients with myelosuppression.

Central nervous system adverse reactions have been reported in a number of patients. These reactions included decreased mental status, exaggerated central nervous system function, and dizziness. More severe obtundation and coma have been rarely observed. Most of these abnormalities were mil and reversible within a few days to three weeks upon dose reduction or discontinuation of Roferon-A therapy. Careful periodic neuropsychiatric monitoring of all patients is recommended.

Leukopenia and elevation of hepatic enzymes occurred frequently but were rarely dose-limiting. Thrombocytopenia occurred less frequently. Proteinuria and increased cells in urinary sediment were also seen infrequently. Rarely, significant hepatic, renal and myelosuppressive toxicities were noted.

PRECAUTIONS: *General:* In all instances where the use of Roferon-A is considered for chemotherapy, the physician must evaluate the need and usefulness of the drug against the risk of adverse reactions. Most adverse reactions are reversible if detected early. If severe reactions occur, the drug should be reduced in dosage or discontinued and appropriate corrective measures should be taken according to the clinical judgment of the physician. Reinstitution of Roferon-A therapy should be carried out with caution and with adequate consideration of the further need for the drug and alertness as to possible recurrence of toxicity.

The minimum effective dose of Roferon-A for treatment of hairy cell leukemia has not been established.

Information for Patient. Patients should be cautioned not to change brands of Interferon without medical consultation, as a change in dosage may result. Patients should be informed regarding the potential benefits and risks attendant to the use of Roferon-A. If home use is determined to be desirable by the physician, instructions on appropriate use should be given, including review of the contents of the enclosed Patient Information Sheet. Patients should be well hydrated, especially during the initial stages of treatment.

Laboratory Tests: Periodic complete blood counts and liver function tests should be performed during the course of Roferon-A treatment. They should be performed prior to therapy and at appropriate periods during therapy. Since responses of hairy cell leukemia are not generally observed for one to three months after initiation of treatment, very careful monitoring for severe depression of blood cell counts is warranted during the initial phase of treatment.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis and Impairment of Fertility:

Carcinogenesis: Roferon-A has not been tested for its carcinogenic potentia

Mutagenesis: A. Internal studies—Ames tests using six different tester strains, with and without metabolic activation, were performed with Roferon-A up to a concentration of 1920 µg/plate. There was no evidence of mutagenicity.

Human lymphocyte cultures were treated *in vitro* with Roferon-A at noncytotoxic concentrations. No increase in the incidence of chromosomal damage was noted.

B. Published studies—There are no published studies on the mutagenic potential of Roferon-A. However, a number of studies on the genotoxicity of human leukocyte interferon have been reported.

A chromosomal defect following the addition of human leukocyte interferon to lymphocyte cultures from a patient suffering from a lymphoproliferative disorder has been reported.

In contrast, other studies have failed to detect chromosomal abnormalities following treatment of lymphocyte cultures from healthy volunteers with human leukocyte interferon.

It has also been shown that human leukocyte interferon protects primary chick embryo fibroblasts from chromosomal aberrations produced by gamma rays.

Impairment of Fertility: Roferon-A has been studied for its effect on fertility in Macaca mulatta (rhesus monkeys). Nonpregnant rhesus females treated with Roferon-A at doses of 5 and 25 million IU/kg/day have shown menstrua cycle irregularities, including prolonged or shortened menstrual periods and erratic bleeding; these cycles were considered to be anovulatory. These monkeys returned to a normal menstrual rhythm following discontinuation o treatment.

Drug Interactions: Interactions between Roferon-A and other drugs have no been fully evaluated.

EGNANCY: *Teratogenic Effects:* Pregnancy Category C. Roferon-A has en shown to demonstrate a statistically significant increase in abortifacient tivity in rhesus monkeys when given at approximately 20 to 500 times the man dose. A study in pregnant rhesus monkeys treated with 1, 5 or 25 lion IU/kg/day of Roferon-A in their early to midfetal period (days 22 to 70 gestation) has failed to demonstrate teratogenic activity for Roferon-A. ere are no adequate and well-controlled studies in pregnant women. *nteratogenic Effects:* Dose-related abortifacient activity was observed in egnant rhesus monkeys treated with 1, 5 or 25 million IU/kg/day of feron-A in their early to midfetal period (days 22 to 70 of gestation). A e-fetal period study (days 79 to 100 of gestation) is in progress and as yet ere have been no reports of any increased rate of abortion. *age in Pregnancy:* Safe use in human pregnancy has not been estab- hed. Therefore, Roferon-A should be used during pregnancy only if the tential benefit justifies the potential risk to the fetus. Information from mate studies showed dose-related menstrual irregularities and an creased incidence of spontaneous abortions. Therefore, fertile women ould not receive Roferon-A unless they are using effective contraception ring the therapy period.

ale fertility and teratologic evaluations have yielded no significant adverse fects to date.

ursing Mothers: It is not known whether this drug is excreted in human lk. Because many drugs are excreted in human milk and because of the tential for serious adverse reactions in nursing infants from Roferon-A, a cision should be made whether to discontinue nursing or to discontinue the ug, taking into account the importance of the drug to the mother.

ediatric Use: Safety and effectiveness in children under 18 years of age ave not been established.

DVERSE REACTIONS: The following data on adverse reactions are ased on the subcutaneous or intramuscular administration of Roferon-A as single agent for the treatment of 63 patients with hairy cell leukemia during vestigational trials in the United States.

u-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), yalgias (73%) and headache (71%) occurred in the majority of patients and nded to diminish with continuing therapy. Other side effects such as norexia (46%), nausea (32%), emesis (10%), diarrhea (29%), dizziness 21%), rash (18%), change in taste (13%), dryness or inflammation of the opharynx (16%), dry skin or pruritus (13%) and weight loss (14%) were oserved with moderate frequency. Less commonly, diaphoresis (8%), aresthesias (6%), numbness (6%), partial alopecia (8%), reactivation of erpes labialis (8%), transient impotence (6%) and arthralgias (5%) were so observed. Rarely (<3%), central nervous system effects including ecreased mental status, depression, visual disturbances, sleep distur- ances and nervousness, as well as cardiac adverse events, including ypertension, chest pain, arrhythmias and palpitations, were reported.

dverse experiences which occurred rarely and may have been related to nderlying disease, included epistaxis, bleeding gums, ecchymosis and etechiae. Miscellaneous adverse events, such as night sweats, urticaria, onjunctivitis and inflammation at the site of injection, were also rarely bserved.

oferon-A has also been evaluated for the treatment of many other types of ancer under investigational trials in the United States. These studies enerally utilized higher doses (12 to 50 million IU/m²). All of the previously escribed adverse reactions which occurred in patients with hairy cell ukemia were also observed in patients receiving higher doses. The inci- ence of most adverse reactions was similar between the two groups, but nded to be more severe in patients who received higher doses of oferon-A. Some additional adverse effects which occurred in these patients cluded confusion (10%), hypotension (6%), lethargy (3%) and edema 3%). Adverse experiences occurring in less than 1% of these patients and bserved only in patients with malignancies other than hairy cell leukemia re as follows: *Gastrointestinal*—abdominal fullness, hypermotility and epatitis; *Central Nervous System*—gait disturbance, poor coordination, hal- cinations, syncope, seizures, encephalopathy, psychomotor retardation, oma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, ysphasia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional labil- y, irritability, hyperactivity, involuntary movements, claustrophobia and loss f libido; *Peripheral Nervous System*—muscle contractions; *Cardiovascular* —congestive heart failure, pulmonary edema, myocardial infarction, aynaud's phenomenon and hot flashes; *Pulmonary*—bronchospasm and achypnea; *Miscellaneous*—excessive salivation and flushing of skin.

bnormal Laboratory Test Values: The percentage of patients with hairy cell eukemia or with other types of malignancies who experienced a significant bnormal laboratory test value at least once during their treatment is shown the following table:

ABNORMAL LABORATORY TEST VALUES

	Hairy Cell Leukemia (n = 63)	Overall Safety Summary* (n = 1019)
ematologic		
eukopenia	59%	69%
eutropenia	39%	58%
hrombocytopenia	42%	42%
ecreased Hemoglobin	36%	6.3%
ecreased Hematocrit	43%	12.5%
ean Time to Nadir		
WBC	38 days	22 days
Platelets	19 days	17 days
epatic		
GOT	47%	78%
lkaline Phosphatase	18%	48%
DH	12%	47%
ilirubin	1.6%	31%
enal/Urinary		
UN	2%	10%
erum Creatinine	3%	10%
ric Acid†	6%	15%
roteinuria†	10%	25%
ther Chemistry Tests		
ypocalcemia	10%	51%
levated FBS‡	33%	39%
levated Serum Phosphorus	2%	17%

*Patients with hairy cell leukemia are included in this overall summary.

†The majority of abnormal uric acid values and proteinuria were of minimal severity. Since these abnormalities are commonly observed in patients with advanced malignancy, it is difficult to ascertain their relationship to Roferon-A treatment.

‡Random glucose determination.

Neutralizing antibodies to Roferon-A were detected in approximately 27% of all patients (3.4% for patients with hairy cell leukemia). No clinical sequelae of their presence have been documented thus far. Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.[4]

DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The induction dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as a subcutaneous or intramuscular injection. Subcutaneous administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count <50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, three times per week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended.

Patients should be treated for approximately six months before the physician determines whether to continue therapy in patients who respond or discontinue therapy in patients who did not respond. Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.

If severe reactions occur, dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve. The minimum effective dose of Roferon-A has not been established.

DIRECTIONS FOR USE: The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are thrombocytopenic (platelet count <50,000) or who are at risk for bleeding.

Injectable Solution:

3 million IU Roferon-A per vial—Each 1 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative.

18 million IU Roferon-A per vial (for multiple-dose use)—Each 1 mL contains 6 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative. Each 0.5 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche.

The injectable solution should be stored in the refrigerator at 36° to 46°F (2° to 8°C). Do *not* freeze. Do not shake.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

HOW SUPPLIED: Sterile Roferon-A is supplied as single and multiple-dose vials:

Roferon-A Injectable Solution: Vials containing 3 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/mL). Boxes of 10 (NDC 0004-1987-01).

Roferon-A Injectable Solution: Multiple-dose vials containing 18 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/0.5 mL). Boxes of 1 (NDC 0004-1988-09).

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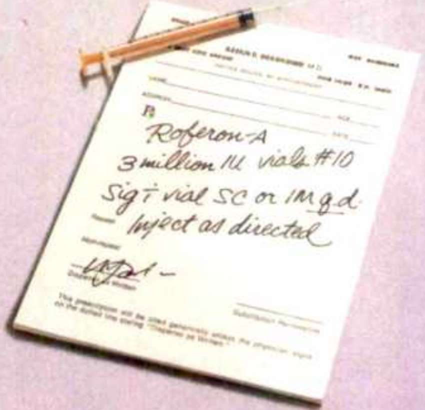

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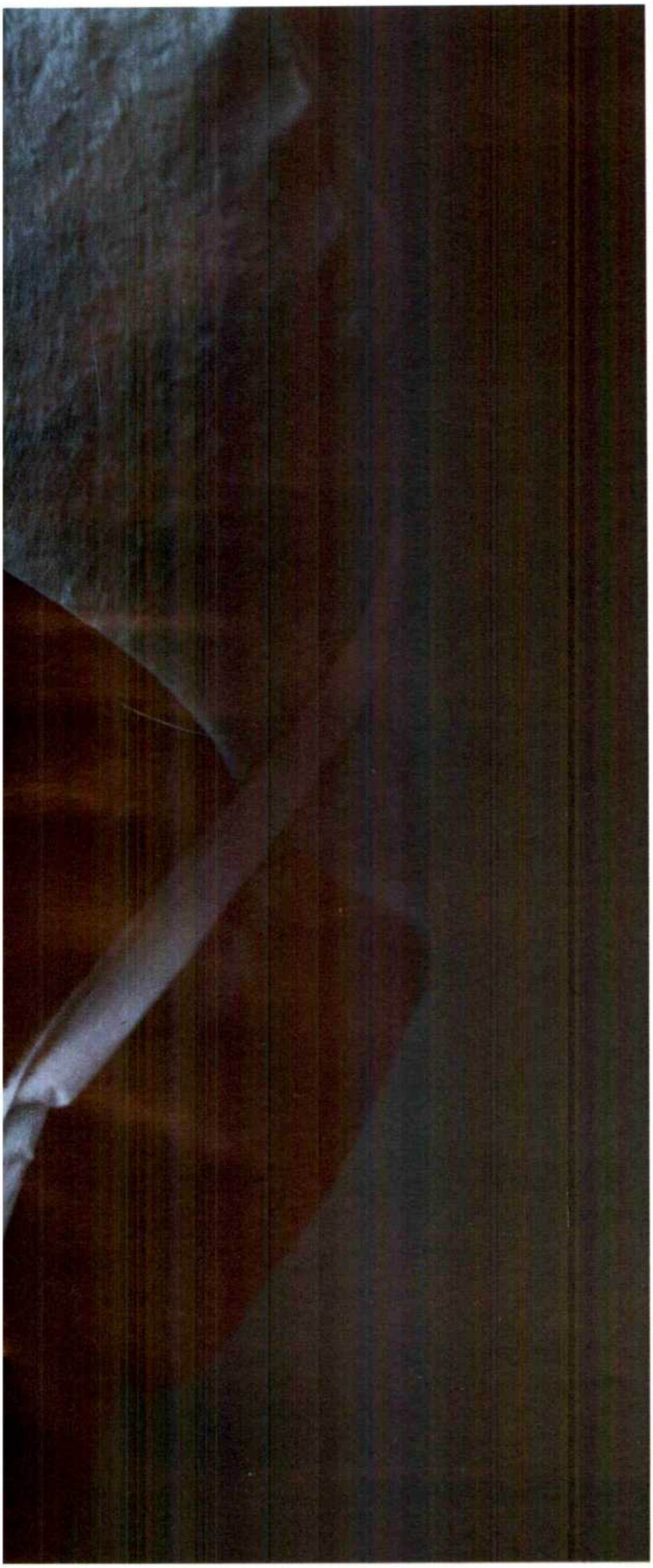

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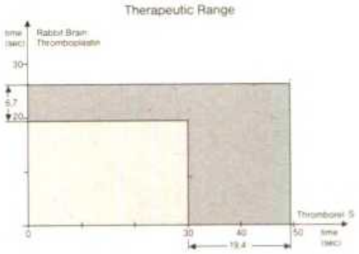

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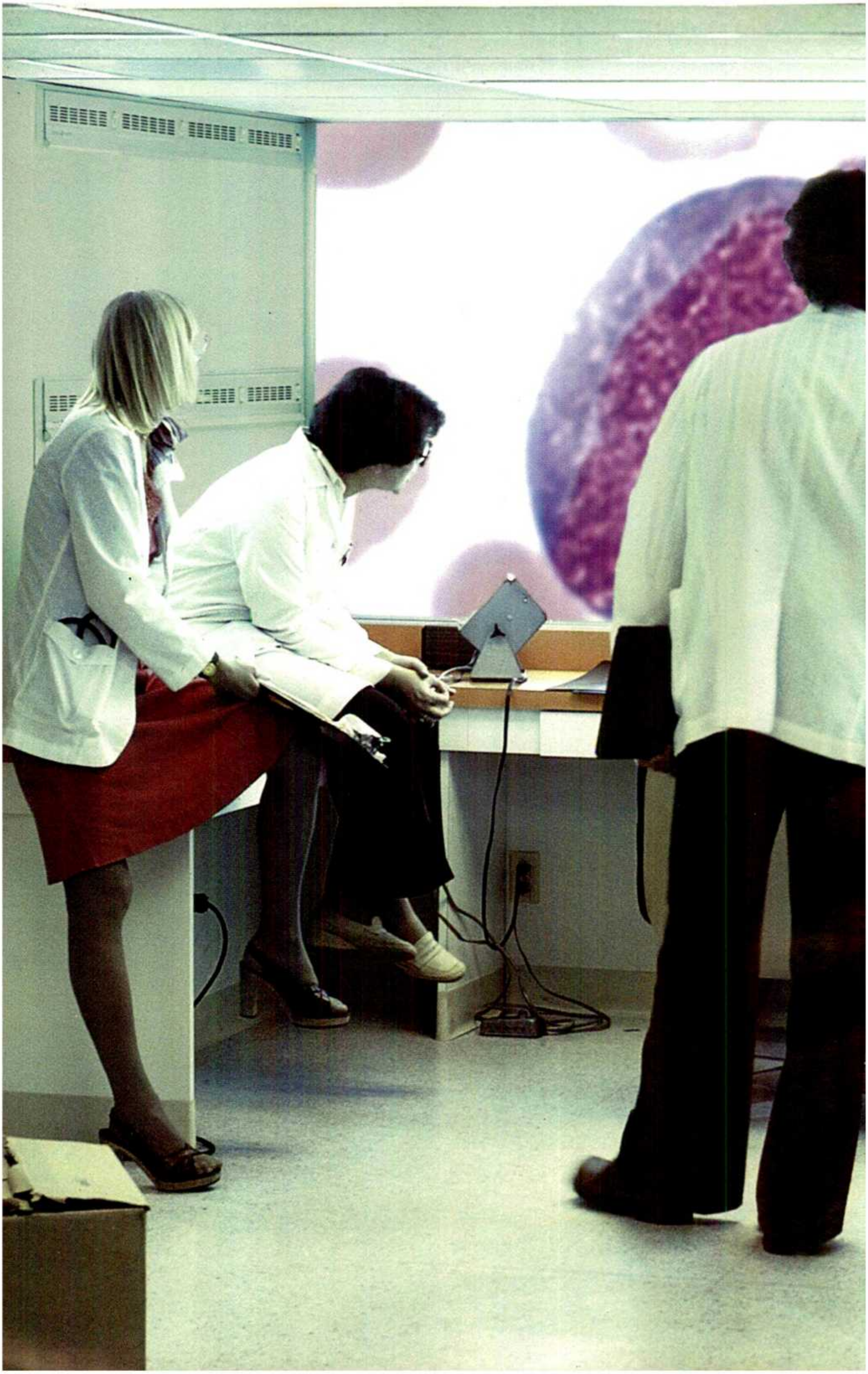

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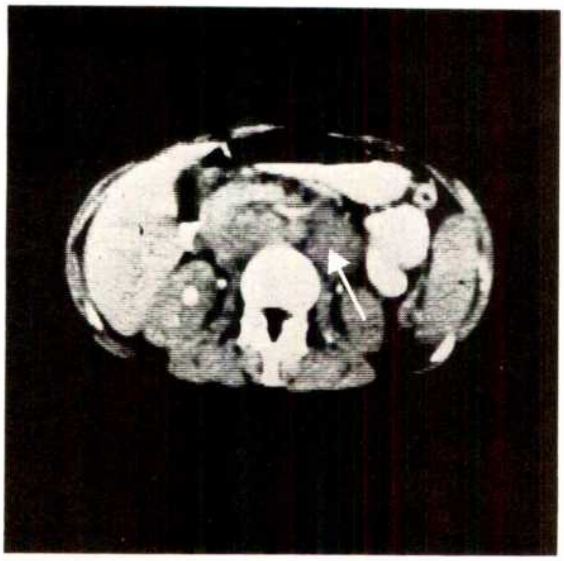
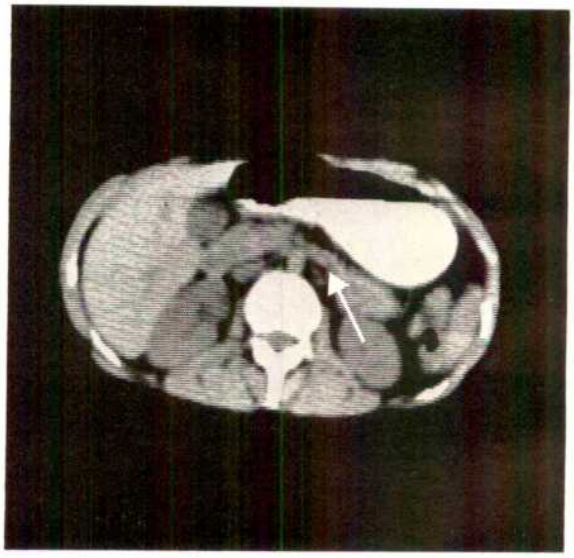


Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

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*Case information on file at Bristol-Myers Oncology Division

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M-BACOD

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MACOP-B

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See prescribing information on following page.

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Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents.

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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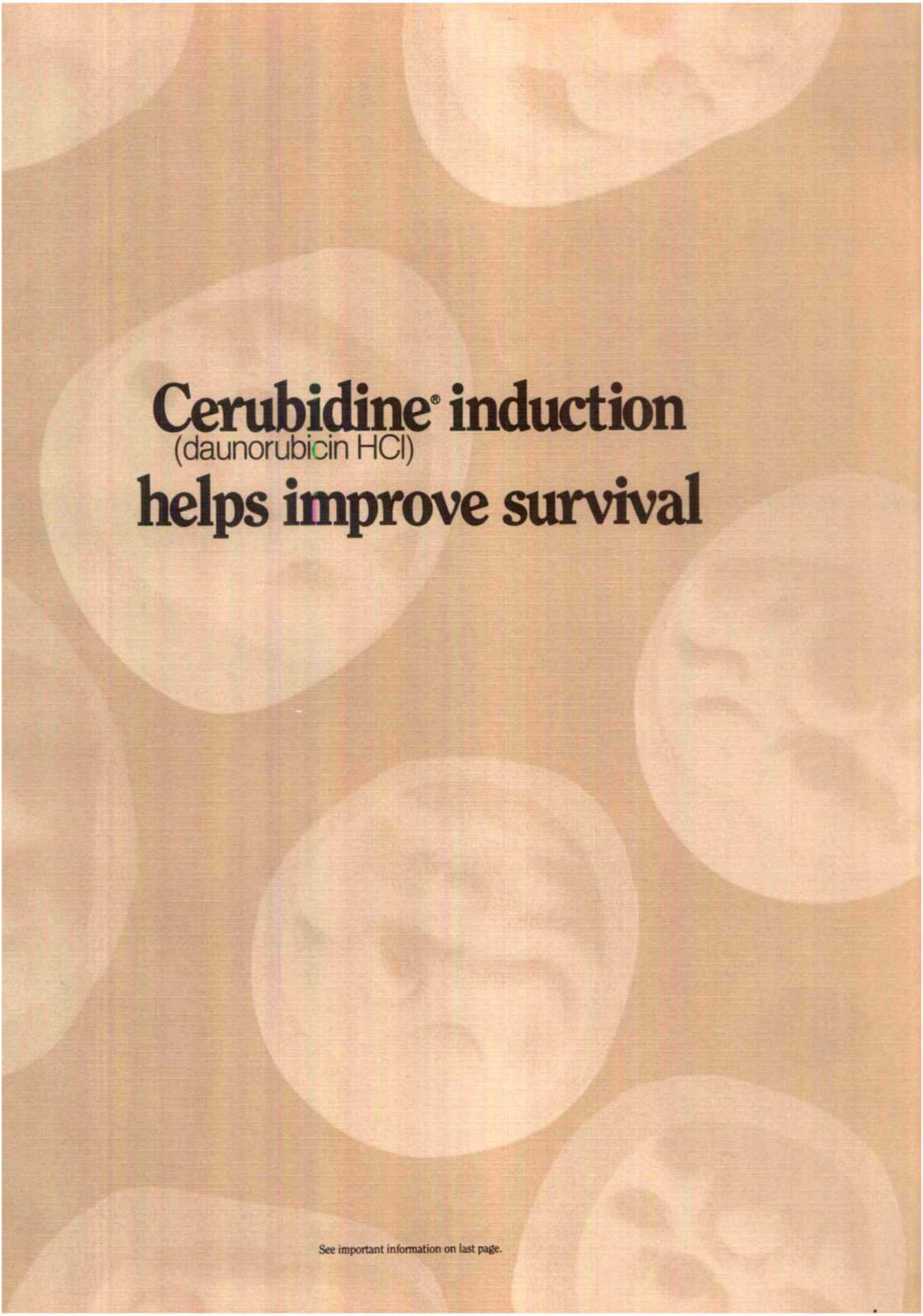

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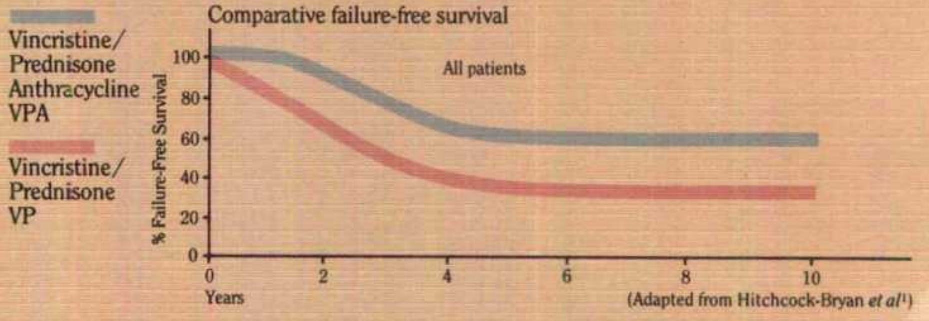

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In combination with ara-C, Cerubidine (45 mg/m^2) yielded 72% complete remissions *vs* 58% for Adriamycin® (doxorubicin) (30 mg/m^2) in combination with ara-C in patients under 60, while a 30 mg/m^2 Cerubidine dosage level produced equal efficacy.[2]

Less catastrophic G.I. toxicity

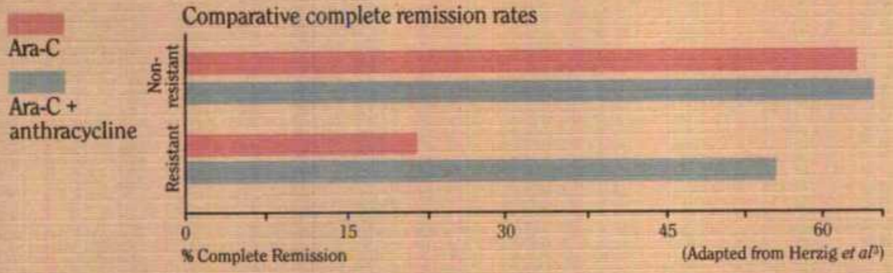
Cerubidine® therapy was associated with lower mortality from necrotizing colitis and was less frequently associated with severe or life-threatening stomatitis.[2]

Cerubidine®
(daunorubicin HCl)

a critical addition to ara-C in reinduction therapy

Complete remissions more than doubled in ara-C resistant patients

(56% *vs* 20%) when an anthracycline was added to a high-dose ara-C reinduction regimen for resistant AML patients.[3]



(Adapted from Herzig *et al*[3])

Less risk of lethal necrotizing colitis

In comparable ara-C + anthracycline regimens, life-threatening necrotizing colitis occurred in 5.6% of patients given Adriamycin® (doxorubicin) compared to 0.23% for those given Cerubidine.[2]

Please see next page for important information.

Cerubidine®
(daunorubicin HCl)

...for survival

Wyeth Laboratories
Philadelphia, Pa 19101

Improves response to initial induction regimens

A critical addition to ara-C in reinduction therapy

Less risk of lethal necrotizing colitis

Cerubidine®

(daunorubicin HCl)

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Cerubidine® (daunorubicin HCl)

for remission induction in acute nonlymphocytic leukemia (myelogenous, monocytic, erythroid) in adults.

WARNINGS

1. Cerubidine® must be given into a rapidly flowing intravenous infusion. It must *never* be given by the intramuscular or subcutaneous route. Severe local tissue necrosis will occur if there is extravasation during administration.
2. Myocardial toxicity manifested in its most severe form by potentially fatal congestive heart failure may be encountered when total cumulative dosage exceeds 550 mg/m² in adults, 300 mg/m² in children more than two years of age; or 10 mg/kg in children less than two years of age.
This may occur either during therapy or several months after termination of therapy. Treatment with digitalis, diuretics, sodium restriction, and bed rest is indicated.
3. Severe myelosuppression occurs when used in therapeutic doses.
4. It is recommended that Cerubidine be administered only by physicians who are experienced in leukemia chemotherapy and in facilities with laboratory and supportive resources adequate to monitor drug tolerance and protect and maintain a patient compromised by drug toxicity. The physician and institution must be capable of responding rapidly and completely to severe hemorrhagic conditions, and/or overwhelming infection.
5. Dosage should be reduced in patients with impaired hepatic or renal function.

Warnings: Therapy should not be started in patients with preexisting drug-induced bone marrow suppression unless the benefit warrants the risk. Pre-existing heart disease and previous therapy with doxorubicin increase the risk of Cerubidine-induced cardiac toxicity; the benefit-to-risk ratio should be weighed before starting Cerubidine in such patients.

Bone Marrow—Cerubidine® is a potent bone marrow suppressant. Suppression will occur in all patients given a therapeutic dose of this drug.

Cardiac Effects[1-6]—Special attention must be given to the potential cardiac toxicity of Cerubidine, particularly in infants and children. *In adults* at total cumulative doses less than 550 mg/m², acute congestive heart failure is seldom encountered. However, rare instances of pericarditis-myocarditis, not dose-related, have been reported.

In adults, at cumulative doses exceeding 550 mg/m², there is an increased incidence of drug-induced congestive heart failure. Based on prior clinical experience with doxorubicin, this limit appears lower, namely 400 mg/m², in patients who received radiation therapy that encompassed the heart.

In infants and children, there appears to be a greater susceptibility to anthracycline-induced cardiotoxicity compared to that in adults, which is more clearly dose-related. However, there is very little risk for children over two years of age in developing Cerubidine-related cardiotoxicity below a cumulative dose of 300 mg/m² or in children less than two years of age (or <0.5 m² body surface area) below a cumulative dose of 10 mg/kg. In both children and adults, the total dose of Cerubidine administered should also take into account any previous or concomitant therapy with other potentially cardiotoxic agents or related compounds such as doxorubicin.

There is absolutely no reliable method of predicting the patients in whom acute congestive heart failure will develop as a result of the cardiac toxic effect of Cerubidine. However, certain changes in the electrocardiogram and a decrease in the systolic ejection fraction from pre-treatment baseline may help to recognize those patients at greatest risk to develop congestive heart failure. On the basis of the electrocardiogram, a decrease equal to or greater than 30% in limb lead QRS voltage has been associated with a significant risk of drug-induced cardiomyopathy. Therefore, an electrocardiogram and/or determination of systolic ejection fraction should be performed before each course of Cerubidine. In the event that one or the other of these predictive parameters should occur, the benefit of continued therapy must be weighed against the risk of producing cardiac damage.

Early clinical diagnosis of drug-induced congestive heart failure appears to be essential for successful treatment with digitalis, diuretics, sodium restriction, and bed rest.

Evaluation of Hepatic and Renal Function—Since significant hepatic or renal impairment can enhance the toxicity of the recommended doses of Cerubidine, hepatic and renal functions should be evaluated with conventional clinical laboratory tests prior to administration.

Pregnancy—Cerubidine® can cause fetal harm when administered to a pregnant woman; patients using this drug during pregnancy, or who become pregnant while taking this drug, should be apprised of the potential hazard to the fetus.

Extravasation at Injection Site—Extravasation of Cerubidine at the site of intravenous administration can cause severe local tissue necrosis.

Precautions: Therapy with Cerubidine requires close observation of the patient and extensive chemical and laboratory monitoring. Hyperuricemia secondary to rapid lysis of leukemic cells may be induced; blood uric acid levels should be monitored and appropriate therapy initiated if hyperuricemia develops. Appropriate measures must be taken to control any systemic infection before beginning therapy with Cerubidine. Therapy with Cerubidine may transiently impart red coloration to the urine after administration, and patients should be advised to expect this.

Carcinogenesis, mutagenesis, impairment of fertility: Cerubidine® when injected subcutaneously into mice, causes fibrosarcomas to develop at the injection site. When administered to mice orally or intraperitoneally, no carcinogenic effect was noted after 22 months of observation.
In male dogs at a daily dose of 0.25 mg/kg administered intr venously, testicular atrophy was noted at autopsy. Histolog examination revealed total aplasia of the spermatocyte seri in the seminiferous tubules with complete aspermatogenesi

Pregnancy Category D: See **Warnings** Section.

Adverse Reactions: Dose-limiting toxicity includes myelosuppression and cardiotoxicity (see **Warnings**). Other reactions include: *Cutaneous*—Reversible alopecia in most patients. *Gastrointestinal*—Acute nausea and vomiting, usually mild, and antiemetic therapy may be of some help; mucositis, thre to seven days after administration; diarrhea. *Local*—If extravasation occurs during administration, tissue necrosis can result at the site. *Acute Reactions*—Fever, chills, skin rash.

Consult direction circular before prescribing.

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INFORMATION FOR CONTRIBUTORS

SUBMITTING THE MANUSCRIPT

BLOOD, The Journal of The American Society of Hematology, provides an international forum for the publication of original articles, in English, describing basic laboratory and clinical investigations encompassed in the broad discipline of hematology. The scope of the journal covers all aspects of hematology, including disorders of leukocytes, both benign and malignant, erythrocytes, platelets, hemostatic mechanisms, and immunology, as well as major developments in clinical laboratory diagnosis and blood banking.

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(Continued on following page)

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Personal communications and references to publications in press by authors other than those submitting the paper must be substantiated by a letter from the investigator(s) concerned confirming the data or observations and granting the authors permission to cite the material.

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11. I forgot to eat this morning.
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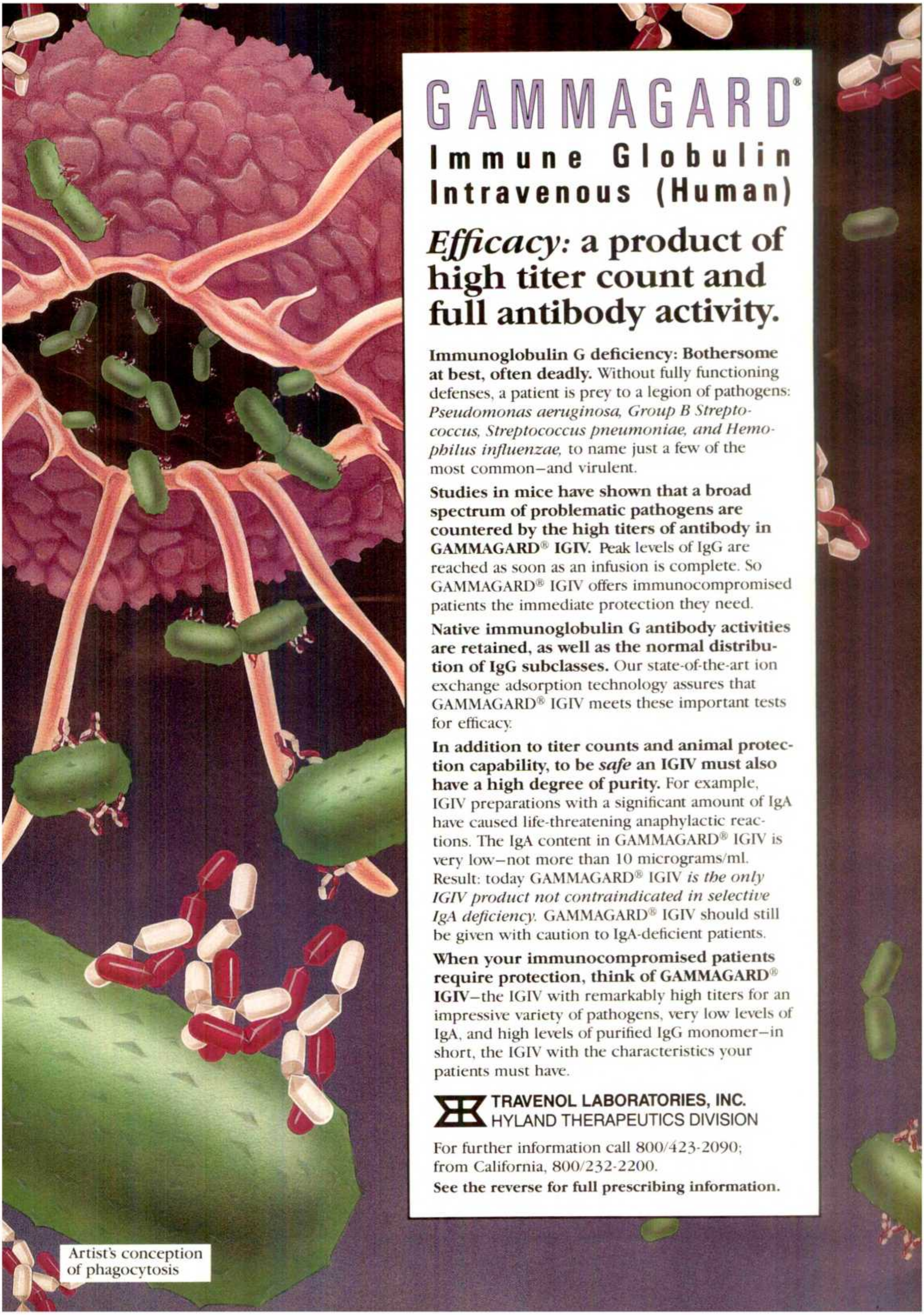

Artist's conception of phagocytosis

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Efficacy: a product of high titer count and full antibody activity.

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In addition to titer counts and animal protection capability, to be *safe* an IGIV must also have a high degree of purity. For example, IGIV preparations with a significant amount of IgA have caused life-threatening anaphylactic reactions. The IgA content in GAMMAGARD® IGIV is very low–not more than 10 micrograms/ml. Result: today GAMMAGARD® IGIV *is the only IGIV product not contraindicated in selective IgA deficiency.* GAMMAGARD® IGIV should still be given with caution to IgA-deficient patients.

When your immunocompromised patients require protection, think of GAMMAGARD® IGIV–the IGIV with remarkably high titers for an impressive variety of pathogens, very low levels of IgA, and high levels of purified IgG monomer–in short, the IGIV with the characteristics your patients must have.

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See the reverse for full prescribing information.

DIRECTION INSERT
Immune Globulin Intravenous (Human) GAMMAGARD®

DESCRIPTION

Immune Globulin Intravenous (Human), GAMMAGARD® * is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8±0.4.

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average, the distribution of IgG subclasses present in this product is the same as is present in normal plasma.[1] Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from plasma units found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum levels is, therefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum proteins.[2,3] Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.[2,3,4,5]

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5]

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

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Inquiries:
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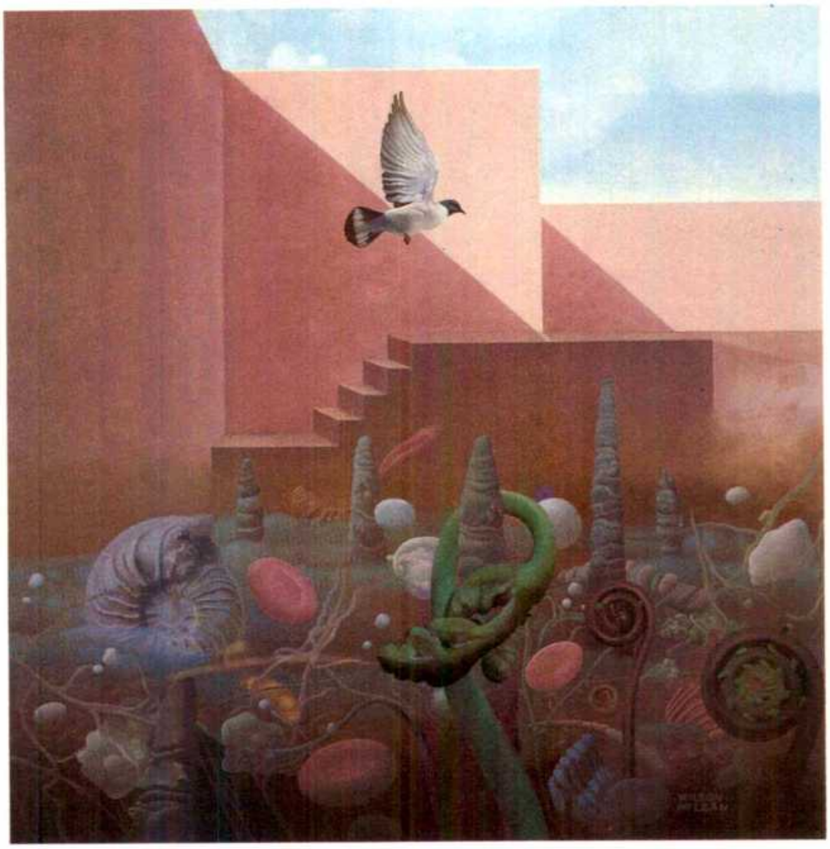

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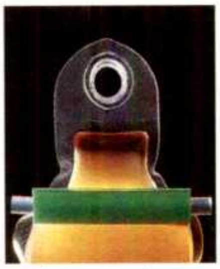

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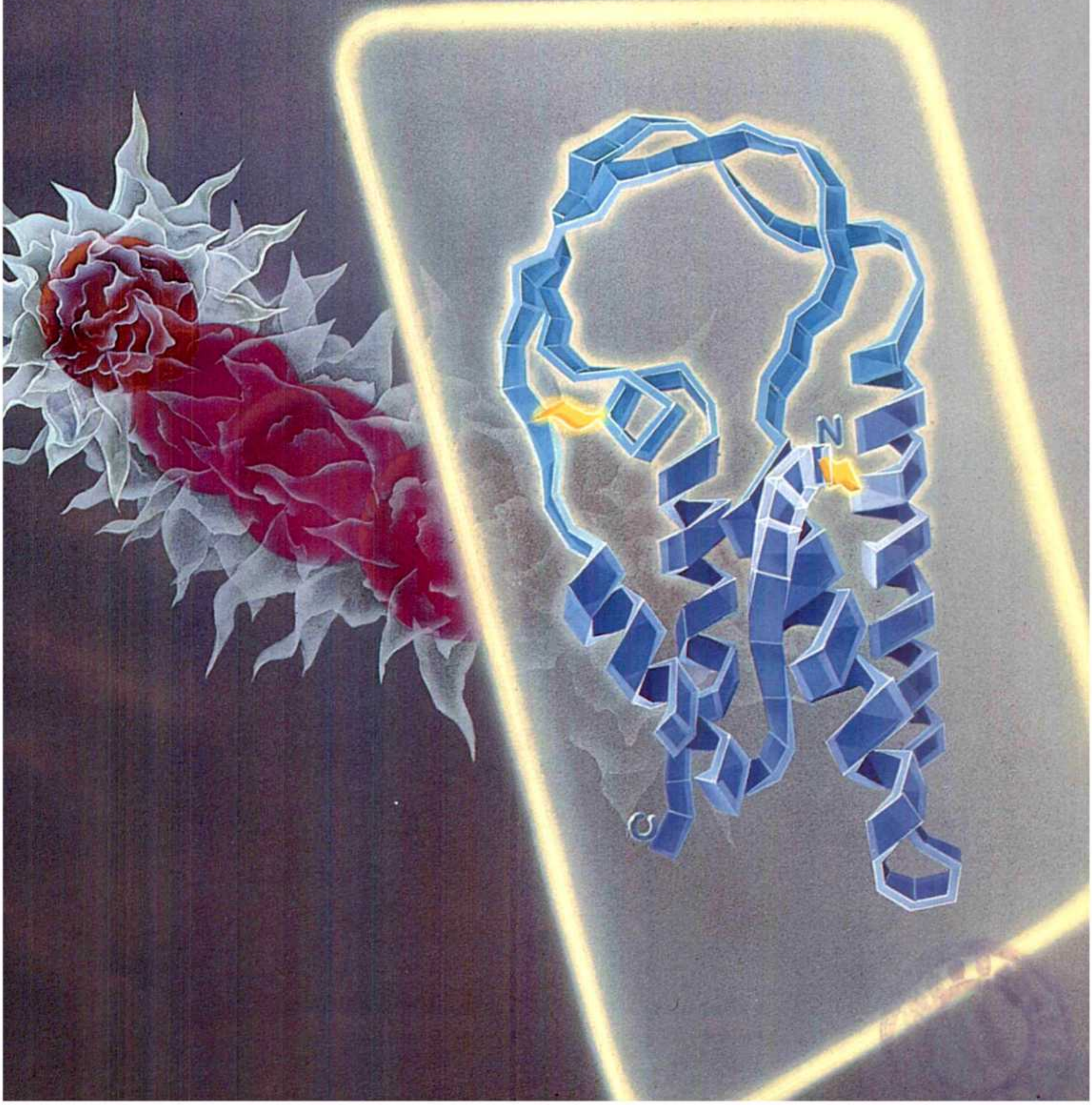
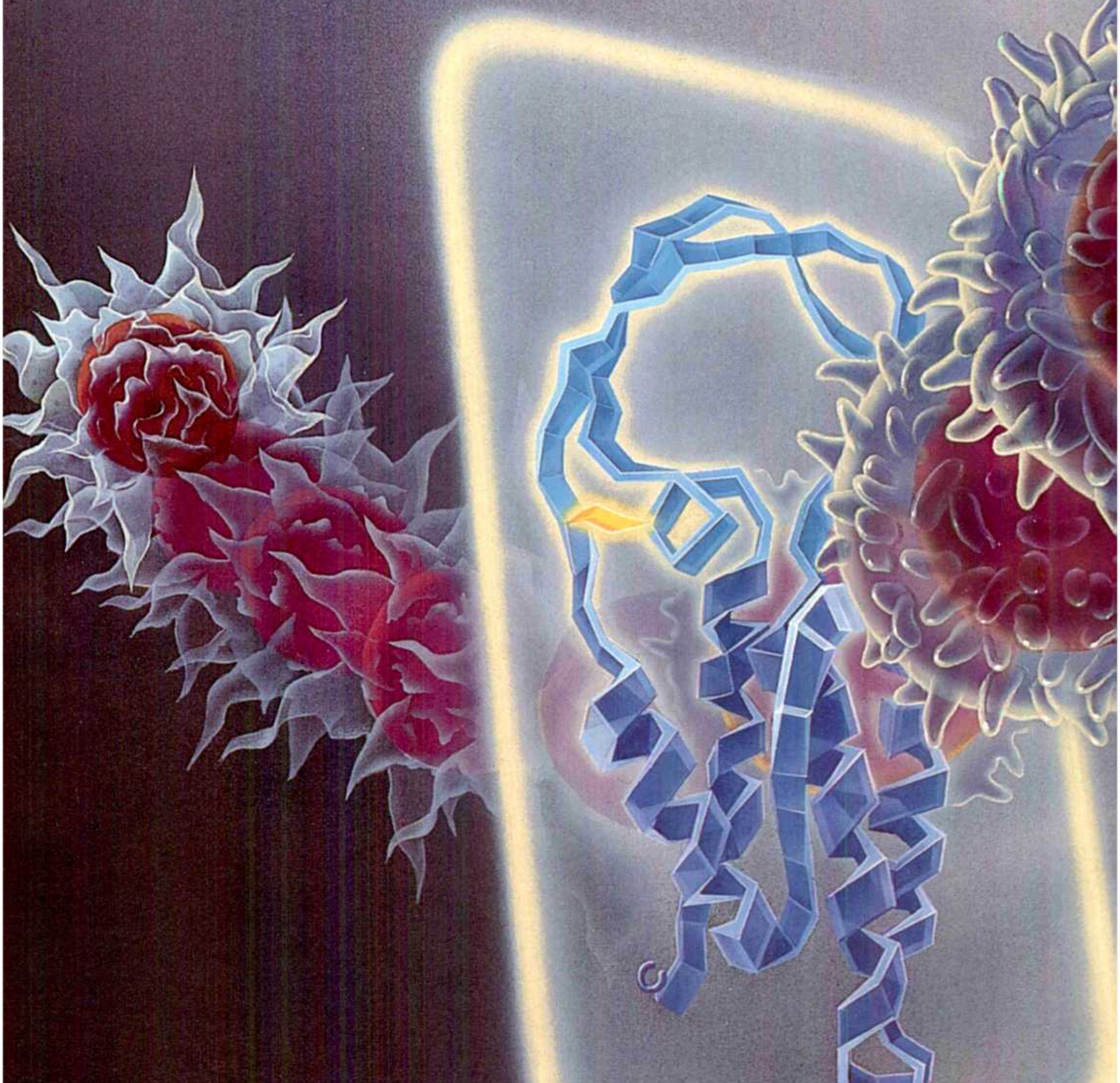
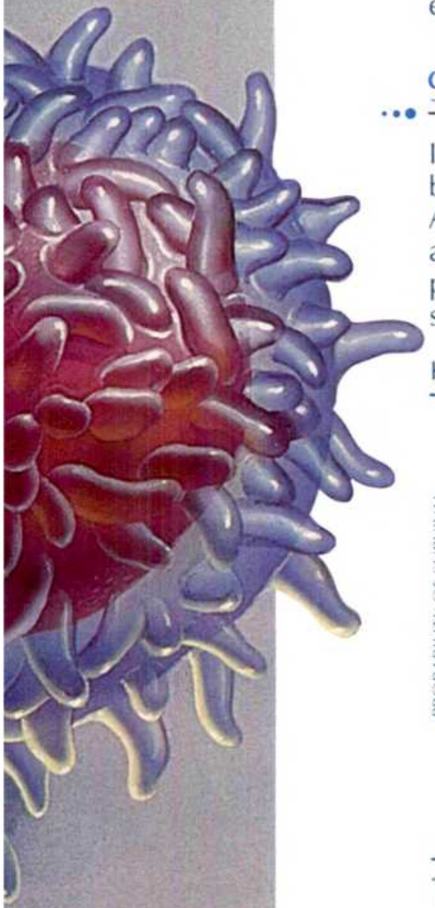


NEW INTRON® A

INTERFERON ALFA-2b, RECOMBINANT INJECTION

CHANGING THE COURSE OF HAIRY CELL LEUKEMIA





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The efficacy of INTRON A was established in a 26-center clinical trial of 145 patients with progressive hairy cell leukemia, a B-cell malignancy.[1,2,3] INTRON A achieved a 74% objective response, measured as normalization of granulocyte, hemoglobin and platelet values and elimination of transfusion dependency.[1]

92% PROBABILITY OF SURVIVAL

In a separate analysis, a 24-month comparison was conducted between the INTRON A study population and historical controls.* After three months of therapy, patients receiving INTRON A reached and maintained a plateau of 92% probability of survival. The probability of survival among historical controls, however, declined steadily to 40% at 24 months.[1]

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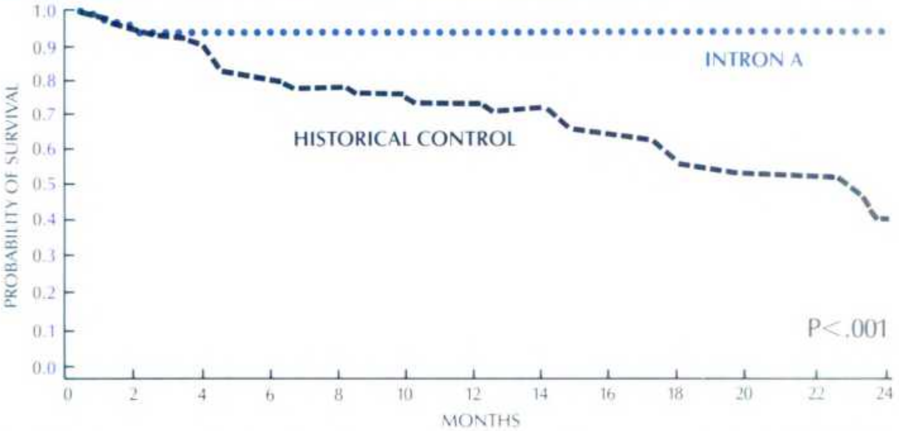


*Seventy-one historical control patients were treated with current standard therapies, 85% with chemotherapy (mainly chlorambucil) and 15% with supportive care.

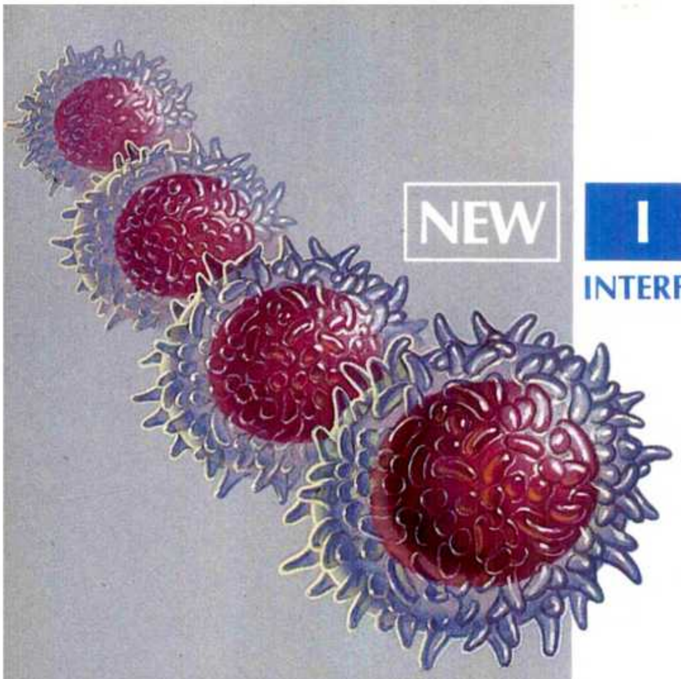
DRAMATIC CLINICAL IMPROVEMENTS

More than 80% of the study population achieved normal levels of platelets by three months, of hemoglobin by six months and of granulocytes by twelve months. Marked decreases in the incidence of life-threatening infections and transfusion dependency preceded normalization of hematologic values.[1]

THERAPY WELL TOLERATED

Treatment-related side effects were generally mild to moderate and rapidly reversible upon discontinuation of therapy. The predominant complaint was a flu-like syndrome, which usually diminished with continued therapy. Mild-to-moderate skin rashes occurred intermittently in 40% of the study population. A low incidence of mild-to-moderate GI disorders was also reported.[1]

For a brief summary of prescribing information, please see the following page.



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INTRON® A Interferon alfa-2b recombinant for Injection

BRIEF SUMMARY

INDICATIONS AND USAGE INTRON A for Injection is indicated for the treatment of patients 18 years of age or older with hairy cell leukemia. Studies have shown that INTRON A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and non-splenectomized patients.

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically during treatment to determine whether response to treatment has occurred. If a patient does not respond within 6 months, treatment should be discontinued. If a response to treatment does occur, treatment usually should be continued until no further improvement is observed and these laboratory parameters have been stable for about 3 months (see **DOSAGE AND ADMINISTRATION**). It is not known whether continued treatment after that time point is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS INTRON A is contraindicated in patients with a history of hypersensitivity to interferon alfa or any component of the injection.

WARNINGS Moderate to severe adverse experiences may require modification of the patient's dosage regimen, or in some cases, termination of therapy with INTRON A.

Because of the fever and other "flu-like" symptoms associated with INTRON A administration, it should be used cautiously in patients with debilitating medical conditions, such as those with a history of cardiovascular disease (e.g., unstable angina, uncontrolled congestive heart failure), pulmonary disease (e.g., chronic obstructive pulmonary disease), or diabetes mellitus prone to ketoacidosis. Caution should also be observed in patients with coagulation disorders (e.g., thrombophlebitis, pulmonary embolism) or severe myelosuppression.

Patients with platelet counts of less than 50,000/mm^3 should not be administered INTRON A intramuscularly, but instead by subcutaneous administration.

Cardiovascular adverse experiences which include significant hypotension, arrhythmia, or tachycardia of 150 beats per minute or greater, were observed in approximately 3% of the patients studied who had various malignancies and were treated at doses higher than those for hairy cell leukemia. The incidence of these complications in patients with preexisting heart disease is unknown. Hypotension may occur during administration, or up to two days post therapy, and may require supportive therapy including fluid replacement to maintain intravascular volume. Supraventricular arrhythmias occurred rarely and appeared to be correlated with preexisting conditions, and prior therapy with cardiotoxic agents. These adverse experiences were controlled by modifying the dose or discontinuing treatment, but may require specific additional therapy.

Those patients with a recent history of myocardial infarction and/or previous or current arrhythmic disorder, who require INTRON A therapy, should be closely monitored (see **Laboratory Tests**).

Central nervous system effects manifested by depression, confusion and other alterations of mental status were observed in about 2% of hairy cell leukemia patients treated with INTRON A. The overall incidence in a larger patient population with other malignancies treated with higher doses of INTRON A was 10%. More significant obtundation and coma have been observed in some patients, usually elderly, treated at higher doses for other malignant diseases. These effects are usually rapidly reversible. In a few severe episodes, full resolution of symptoms has taken up to three weeks. Patients should be closely monitored until resolution of these effects. Discontinuation of INTRON A Interferon alfa-2b, recombinant for Injection therapy may be required. Narcotics, hypnotics, or sedatives may be used concurrently with caution.

Acute serious hypersensitivity reactions (e.g., urticaria, angioedema, bronchoconstriction, anaphylaxis, etc.) have not been observed in patients receiving INTRON A; however, if such an acute reaction develops, the drug should be discontinued immediately and appropriate medical therapy instituted. Transient cutaneous rashes have occurred in some patients following injection, but have not necessitated treatment interruption.

Laboratory abnormalities which occurred in hairy cell leukemia patients included elevated SGOT and SGPT, which occurred in 4% and 13% of patients, respectively. The overall incidences of laboratory abnormalities in a larger patient population with other malignancies, and treated at higher doses were somewhat higher. These included elevated liver function tests (SGOT, SGPT in 10% of patients) and reductions in granulocyte (20% of patients) and platelet counts (18% of patients) (see **Laboratory Tests**). These abnormalities are usually mild to moderate and transient. Severe abnormalities of these laboratory parameters are usually rapidly reversible upon cessation or reduction of INTRON A therapy.

PRECAUTIONS Information for Patients Patients being treated with INTRON A should be directed in its appropriate use, informed of benefits and risks associated with treatment and referred to the Patient Information Sheet.

Patients should be cautioned not to change brands of Interferon without medical consultation as a change in dosage may result.

It is advised that patients be well hydrated especially during the initial stages of treatment.

Laboratory Tests In addition to those tests normally required for monitoring patients with hairy cell leukemia, the following laboratory tests are recommended for all patients on INTRON A therapy, prior to beginning treatment and then periodically thereafter:

- Standard hematologic tests — including complete blood counts and differential as well as platelet counts.
- Blood chemistries — electrolytes and liver function tests.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer, should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis, Impairment of Fertility Studies with INTRON A have not been performed to determine carcinogenicity, or the effect on fertility. Interferon may impair fertility. Mutagenicity studies with INTRON A revealed no adverse effects.

Studies in mice, rats, and monkeys injected with INTRON A for up to one month have revealed no evidence of toxicity. However, due to the known species-specificity of interferon, the effects in animals are unlikely to be predictive of those in man.

Pregnancy Category C Animal reproduction studies have not been conducted with INTRON A. It is also not known whether INTRON A can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. INTRON A should be given to a pregnant woman only if clearly needed. Another interferon alfa preparation has been shown to have abortifacient effects in *Macaca mulatta* (rhesus monkeys) when given at 20 to 500 times the human dose. Therefore, INTRON A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. In studies of interferon use in non-human primates, abnormalities of the menstrual cycle have been observed.

INTRON A Interferon alfa-2b, recombinant for Injection should be used with caution in fertile men and women.

Nursing Mothers It is not known whether this drug is excreted in human milk. However, studies in mice have shown that mouse interferons are excreted into the milk. Because of the potential for serious adverse reactions from INTRON A in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use Safety and effectiveness have not been established in patients below the age of 18 years.

ADVERSE REACTIONS Adverse reactions to INTRON A are dose-related. In studies of patients with malignancies other than hairy cell leukemia, hematologic and hepatic toxicities were more common in patients receiving individual injections greater than 10 million IU, than in patients receiving less than this amount. Hematologic, hepatic, cardiovascular and neurologic toxicities were more common in patients receiving more than 30 million IU per injection than in patients receiving less than that amount.

The following adverse reactions were reported to be possibly or probably related to INTRON A therapy during clinical trials. The most frequently reported adverse reactions were flu-like symptoms, primarily fever, fatigue, and chills which occurred in almost all patients treated. Other organ systems in which reactions occurred in more than 5% of the patients are:

General — Taste alteration, anorexia and weight decrease occurred uncommonly.

Cardiovascular — Mild hypotension was frequently recorded although it was rarely symptomatic. Hypertension also occurred less commonly. Tachycardias occurred uncommonly usually in association with high fevers.

Gastrointestinal System — Nausea was common, although vomiting occurred rarely. Mild diarrhea was reported less commonly.

Hematologic — Mild thrombocytopenia and transient granulocytopenia occurred commonly.

Musculo-Skeletal System — Myalgia and arthralgia occurred commonly, usually in association with other flu-like symptoms.

Nervous System — Headache, somnolence, confusion, and dizziness occurred uncommonly. Ataxia and paresthesia were rare.

Psychiatric — Anxiety, depression and nervousness occurred uncommonly.

Skin and Appendages — Mild pruritus occurred uncommonly. Mild alopecia was uncommon. Various transient, mild skin rashes were seen frequently in patients with hairy cell leukemia.

Other Adverse Reactions — The following adverse reactions were reported with an incidence of 5% or less: leg cramps, constipation, insomnia, herpetic eruptions, non-herpetic cold sores, urticaria, hot flashes, supraventricular arrhythmias, epistaxis, stomatitis, paralytic ileus, dehydration, coagulation disorder (elevated PT and PTT), abnormal vision, tremor, emotional lability, chest pain, pharyngitis and syncope.

Adverse reactions reported rarely (less than 1%) were dyspepsia, purpura, dyspnea, sneezing, oculomotor paralysis, nasal congestion, flatulence, increased saliva, hyperglycemia, and ulcerative stomatitis.

Laboratory Value Changes — Those patient laboratory values which were normal to moderately abnormal (WHO grades 0–2) at baseline that worsened to either severe or life-threatening abnormalities (WHO grades 3 or 4) during some phase of treatment included: WBC, platelets, granulocytes, SGOT, creatinine, SGPT, LDH, and alkaline phosphatase.

DOSAGE AND ADMINISTRATION The recommended dosage of INTRON A for Injection for the treatment of hairy cell leukemia is 2 million IU/m^2 administered intramuscularly (see **WARNINGS**) or subcutaneously 3 times a week. Higher doses are not recommended. The normalization of one or more hematologic variables usually begins within 2 months of initiation of therapy. Improvement in all three hematologic variables may require 6 months or more of therapy.

This dosage regimen should be maintained unless the disease progresses rapidly, or severe intolerance is manifested. If severe adverse reactions develop, the dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. If persistent or recurrent intolerance develops following adequate dosage adjustment, or disease progresses, treatment with INTRON A Interferon alfa-2b, recombinant for Injection should be discontinued. The minimum effective dose of INTRON A has not been established.

At the discretion of the physician, the patient may self-administer the dose. The medication may be administered at bedtime.

HOW SUPPLIED INTRON A Interferon alfa-2b, recombinant for Injection is available in 3-, 5-, 10- and 25-million IU vials, each packaged with its own diluent.

Store INTRON A for Injection, both before and after reconstitution, between 2° and 8°C (36° and 46°F).

JBS-13762805

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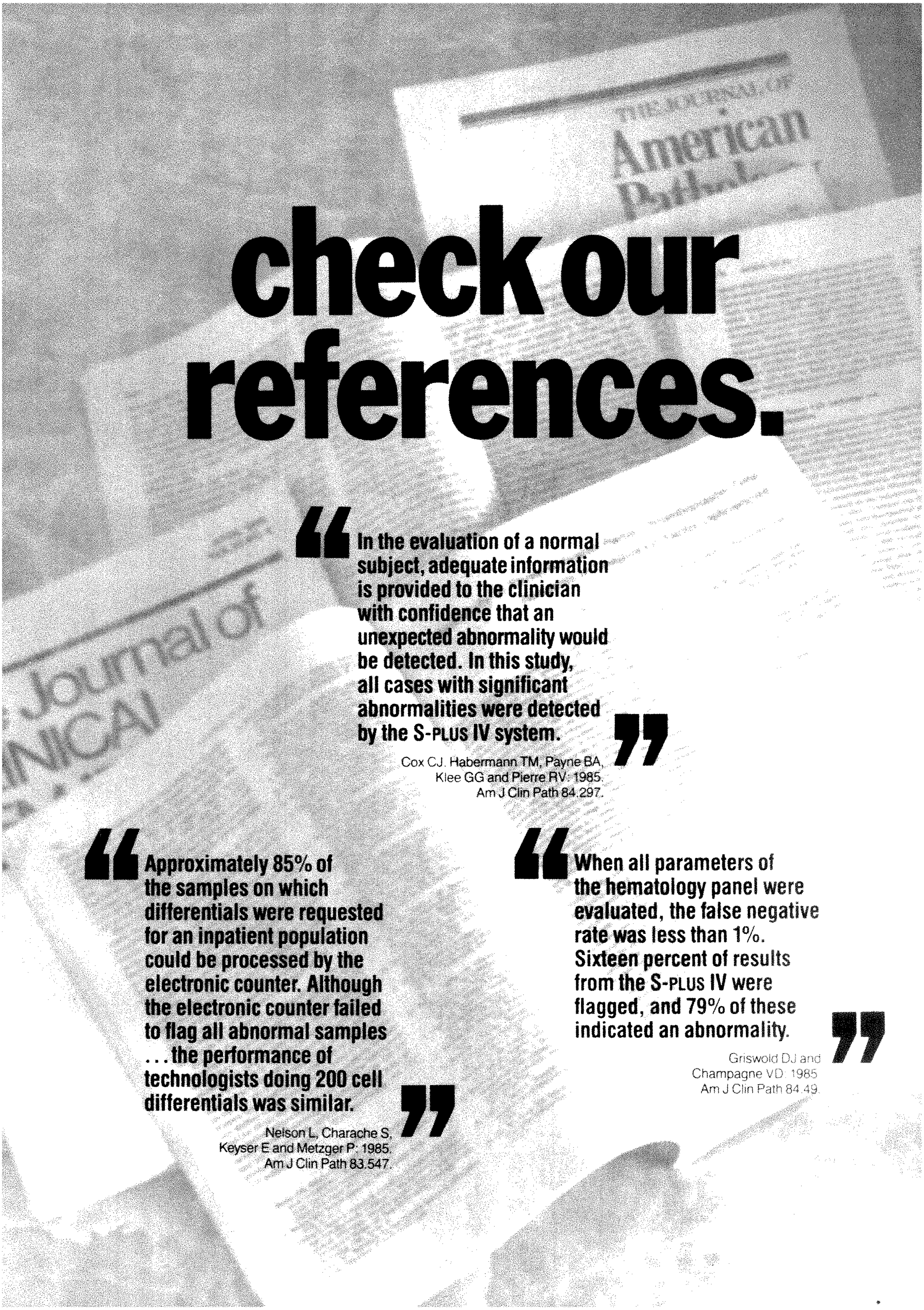

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Cox CJ, Habermann TM, Payne BA,
Klee GG and Pierre RV: 1985.
Am J Clin Path 84:297.

> "Approximately 85% of the samples on which differentials were requested for an inpatient population could be processed by the electronic counter. Although the electronic counter failed to flag all abnormal samples . . . the performance of technologists doing 200 cell differentials was similar."

Nelson L, Charache S,
Keyser E and Metzger P: 1985.
Am J Clin Path 83:547.

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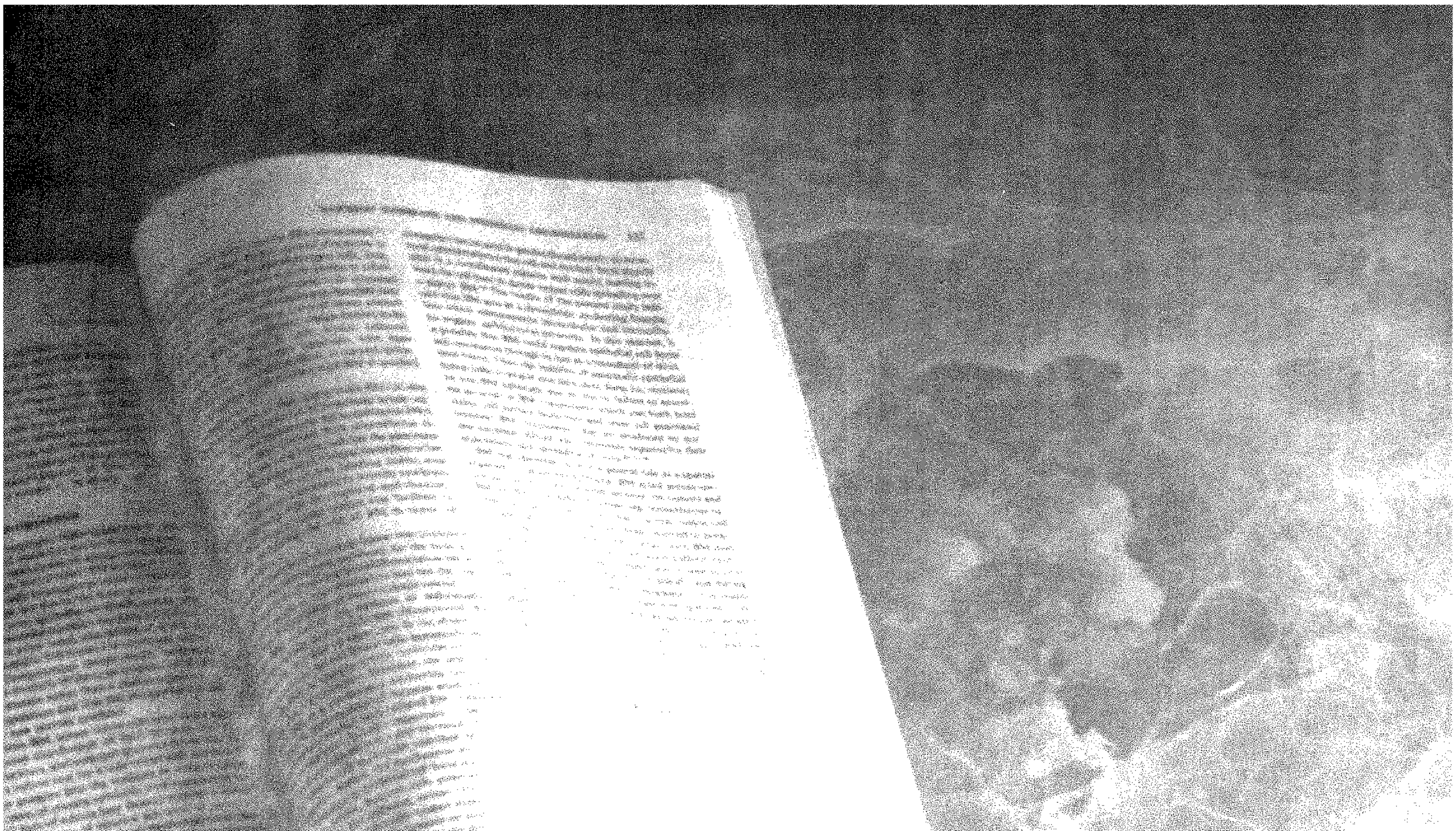
Griswold DJ and
Champagne VD: 1985
Am J Clin Path 84:49.



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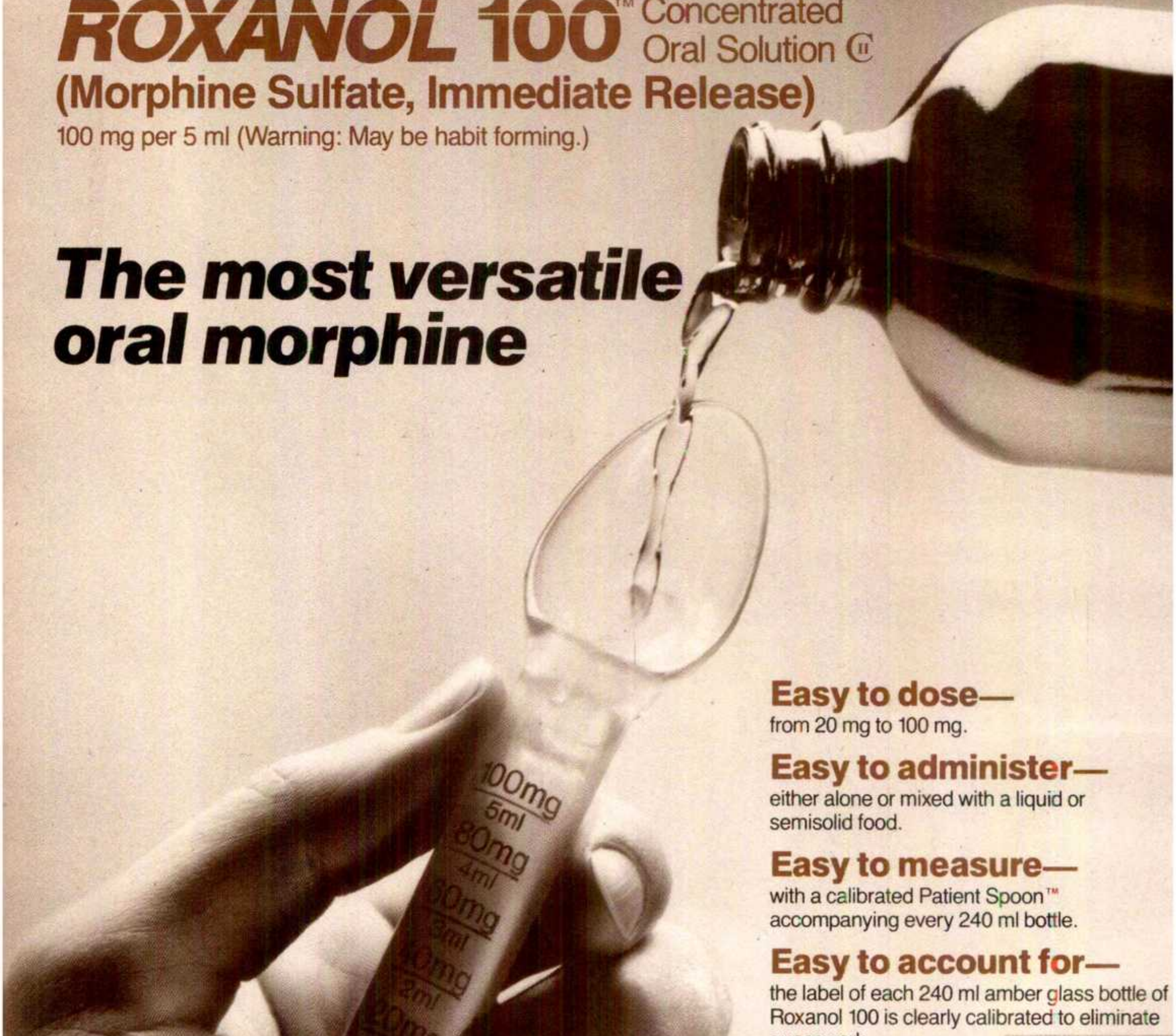

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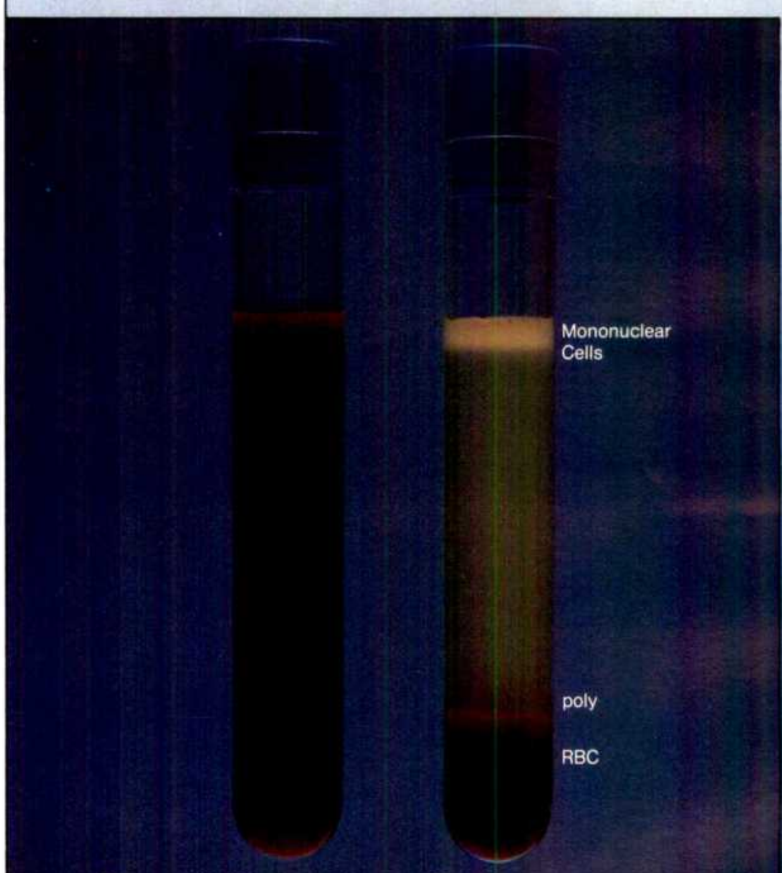

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REVIEW

Light Chains of Immunoglobulins: Structural-Genetic Correlates

By Alan Solomon

WITHIN the past decade, studies by molecular geneticists on the composition and organization of immunoglobulin (Ig)-coding genes at the DNA level have vastly increased our understanding of the genetic mechanisms responsible for generating antibody diversity and for linking Ig synthesis to cellular differentiation. This new information, which has far-reaching biological and clinical implications, has come from research on B cell-related lymphocytic and plasma cell tumors and on their monoclonal Ig products—Bence Jones proteins, myeloma proteins, and Waldenström macroglobulins.

Bence Jones proteins, long recognized as a diagnostic hallmark of multiple myeloma, have a fundamental influence on the pathophysiological manifestations of this disease. Edelman and Gally's[1] seminal discovery, less than 25 years ago, that Bence Jones proteins represent homogeneous counterparts of Ig light polypeptide chains made evident the importance of these components as materials for the study of the immune system and confirmed their prediction that chemical analyses of Bence Jones proteins would provide a characteristic molecular structure for the light chain. This article updates earlier reviews of the subject[2,3] and summarizes current knowledge of human light chain structure and the molecular genetic events involved in light chain synthesis.

STRUCTURAL FEATURES OF HUMAN LIGHT CHAINS

Complementary studies of Ig proteins and genes have shown a characteristic structure of the light chain consisting of a ~107-residue N-terminal half, the variable (V_L) domain (of which the first ~95 residues are encoded by a V gene segment and the remaining ~12 to 13 residues are encoded by a J gene segment); for κ chains, a 107-residue C-terminal half, the constant (C_L) domain, is encoded by a single C_κ gene; for λ chains, a 105-residue C_L domain is encoded by one of multiple C_λ genes. The V_L contains three regions of extensive sequence variability, the hypervariable or complementarity-determining regions (CDRs) that are involved in the antigen-binding and idiotype sites. The remaining portions of the V_L consist of four regions of lesser sequence variability that provide a structural framework for the CDRs and are designated the framework regions (FR). A schematic representation of the light polypeptide chain is shown in Fig 1.

Light chain V region (V_L). The V regions of light chains, V_κ and V_λ, are readily crystallizable, contain the antigen-binding site, and have other properties that reflect individual differences among light chains (eg, idiotypic antigenic determinants, self-associative and thermal properties, etc.).[2,3] Homologies in sequence, especially those in FR1, chemically define four subgroups of κ chains and six subgroups of λ chains[4]; the prototype subgroup sequences are given in Table 1. The four chemically defined κ chain V region subgroups (κI, κII, κIII, and κIV) and five of the six λ chain subgroups (λI, λII, λIII, λIV, and λVI) also have been confirmed serologically (proteins classified chemically as λV react with specific anti-λII antisera).[5] The designation of the human light chain V region subgroups (and C region factors) is given in Table 2.

The V_L subgroups are also isotypes, ie, molecules representative of each subgroup are found among light chains of Ig molecules present in a normal individual's serum. The percentage of Igκ molecules with κI, κII, κIII, and κIV light chains is 56, 10, 30, and 4, respectively. The distribution of λI, λII/λV, λIII, λIV, and λVI chains among Igλ molecules is 26%, 38%, 22%, 3%, and 11%, respectively.[5,6] A physiological role for V_L isotypes is implied by the finding of a preferential association of a sub-subgroup of κIII light chains, κIIIb, with IgMκ autoantibodies (rheumatoid factors, anti-I cold agglutinins, and anti–low-density lipoprotein antibodies),[7,8] and of λVI chains with primary or multiple myeloma-associated amyloidosis.[6,9]

Light chain C region (C_L). The C regions of light chains, C_κ and C_λ, contribute to the disulfide bond covalently linking the light chain to the heavy chain (Fig 1). The limited sequence differences among C_κ are allotype-related, and only

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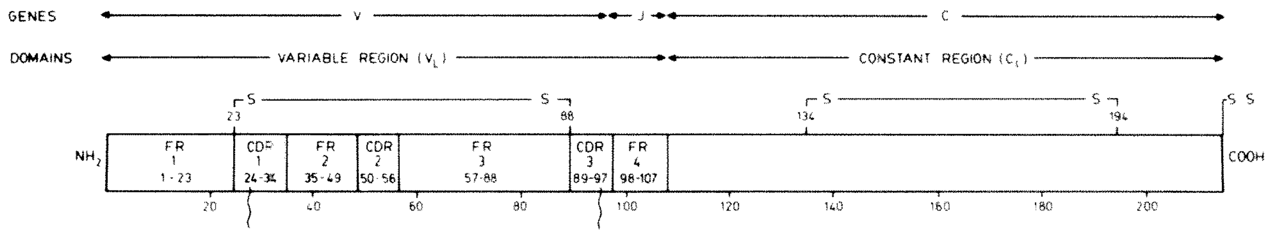

Fig 1. Schematic representation of light chain variable (V_L) and constant (C_L) domains and their genetically-encoded segments. The V_L is the product of two gene segments *V* and *J* that encode, respectively, the first ~95 residues and the remaining ~13 residues of the V region. The C_L is the product of a single gene designated *C* that encodes for the remaining ~106 residues of the light chain. The location of the three hypervariable or complementarity-determining regions (CDRs) and of the four framework regions (FR) are as indicated; the J encoded portion of the V_L encompasses the terminal residue(s) of CDR3 and all the FR4 residues. The wavy line symbolizes the location of additional amino acid residues typically found in CDR1 and at the V-J junction. The residue numbering system is according to ref. 4.

a single isotype has been recognized. Three different allotypes of the C_κ gene are known: These factors (formerly designated *Inv*) are *Km*(1), *Km*(2), and *Km*(3) (Table 2). The three allotypes, inherited in typical Mendelian fashion, are distinguished by sequence differences at two positions (Fig 2) and are also recognized serologically.[2] Lambda chains differ distinctively from κ chains by the extent of C region sequence heterogeneity[10] that is *not* allotype-related but rather is a reflection of at least three isotypes—*Mcg*, *Kern*, and *Oz*.[2] No allotypic markers have been identified as yet on C_λ. The distribution of λ chains with the *Mcg*, *Kern*, and *Oz* isotypes within a normal individual's serum Igλ pool is ~11%, 18%, and 25%, respectively.[10] The functional role of these isotypes is unknown.

Light chain tertiary structure. Much of our knowledge on the tertiary structural features of the Ig molecule has come from x-ray diffraction data obtained from crystals of Fab and V_L fragments and Bence Jones protein dimers.[2,3,11-13] Because of the flexible nature of the "switch" region between V_L and C_L domains, the conformation of a light chain dimer closely resembles that of a Fab fragment.[11] The fact that light chain dimers can function as antibody is evidenced by equilibrium dialysis and crystallographic analyses.[11,14] The structural features of the antigen-binding site are determined primarily by the nature of the CDRs but, as recent data have shown, striking conformational differences in this site can result from the influence of FR residues on V-V domain association.[15-18] The chemical nature of the contacting V_L residues (particularly at position 96) affects the extent of noncovalent interactions between light chain monomers or between heavy and light chains and also affects the configuration of the antigen-binding site.[17,18] Further information on the self-associative properties of light chains and other types of V domain interactions is also essential for understanding the pathophysiological role of these proteins in the light chain-associated diseases.

MOLECULAR GENETICS OF LIGHT CHAINS

Remarkable advances in our knowledge of the molecular genetics involved in Ig synthesis have come from the development of technology involving recombinant DNA, molecular hybridization and cloning, and rapid nucleotide sequence analyses (summarized in ref. 19). These techniques have made it possible to determine the nucleotide sequence at the DNA and RNA level of light chain-specifying genes and to localize, on germline and somatic DNA, the gene segments controlling light chain synthesis. This new information has also shown how light chain gene organization, translocation, and splicing are linked to B cell differentiation and to the generation of antibody diversity. Studies of mouse and human germline DNA (derived from sperm or fetal liver)

Table 1. Prototype Sequences for the First 23 Amino Acids (Framework Region 1) of the Four κ and Six λ Human Light Chain Subgroups

Subgroup	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
κ-Chain																							
κI	Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys
κII	Asp	Ile	Val	Met	Thr	Gln	Ser	Pro	Leu	Ser	Leu	Pro	Val	Thr	Pro	Gly	Glu	Pro	Ala	Ser	Ile	Ser	Cys
κIII	Glu	Ile	Val	Leu	Thr	Gln	Ser	Pro	Gly	Thr	Leu	Ser	Leu	Ser	Pro	Gly	Glu	Arg	Ala	Thr	Leu	Ser	Cys
κIV	Asp	Ile	Val	Met	Thr	Gln	Ser	Pro	Asn	Ser	Leu	Ala	Val	Ser	Leu	Gly	Glu	Arg	Ala	Thr	Ile	Ser	Cys
λ-Chain																							
λI	PCA*	Ser	Val	Leu	Thr	Gln	Pro	Pro	Ser	[ ]†	Val	Ser	Gly	Ala	Pro	Gly	Gln	Arg	Val	Thr	Ile	Ser	Cys
λII	PCA	Ser	Ala	Leu	Thr	Gln	Pro	Ala	Ser	[ ]	Val	Ser	Gly	Ser	Pro	Gly	Gln	Ser	Ile	Thr	Ile	Ser	Cys
λIII	Ser‡	Tyr	Glu	Leu	Thr	Gln	Pro	Pro	Ser	[ ]	Val	Ser	Val	Ser	Pro	Gly	Gln	Thr	Ala	Arg	Ile	Thr	Cys
λIV	—	Ser	Glu	Leu	Thr	Gln	Pro	Pro	Ser	[ ]	Val	Ser	Val	Ala	Pro	Gly	Gln	Thr	Val	Arg	Ile	Thr	Cys
λV	PCA	Ser	Ala	Leu	Thr	Gln	Pro	Pro	Ser	[ ]	Ala	Ser	Gly	Ser	Pro	Gly	Gln	Ser	Val	Thr	Ile	Ser	Cys
λVI	Asn	Phe	Met	Leu	Thr	Gln	Pro	His	Ser	[ ]	Val	Ser	Glu	Ser	Pro	Gly	Lys	Thr	Val	Thr	Ile	Ser	Cys

The amino acid residue occurring exclusively or most often at a designated position is shown. Sequence data from ref. 4.

*PCA represents pyrrolidone-carboxylic acid.

†For maximum homology between κ and λ chain FR1 sequences, a gap is introduced between positions 9 and 11 (ref. 4).

‡This residue is deleted in λIV and some λIII chains.

Table 2. Designation of Human Immunoglobulin Light Chain Variable (V_L) and Constant (C_L) Regions

Light Chain	Variable Region (V_L) Subgroup	Constant Region (C_L)	
		Allotype	Isotype
Kappa (κ)	$V_{\kappa I}$, $V_{\kappa II}$, $V_{\kappa III}$, $V_{\kappa IV}$	*Km(1), Km(1, 2), Km(3)*	0
Lambda (λ)	$V_{\lambda I}$, $V_{\lambda II}$, $V_{\lambda III}$, $V_{\lambda IV}$, $V_{\lambda V}$, $V_{\lambda VI}$	?	$C_{\lambda 1}$ (*Mcg*), $C_{\lambda 2}$ (*Kern*$^-$*Oz*$^-$), $C_{\lambda 3}$ (*Kern*$^-$*Oz*$^+$), $C_{\lambda 4}$, $C_{\lambda 5}$, $C_{\lambda 6}$ . . .

and of somatic DNA (derived from plasmacytomas and other B cell-related neoplasms) have revealed that embryonic light chain-specifying genes are noncontiguous (intergenic untranslated sequences of nucleotide bases are termed intervening sequences or introns, as opposed to the translated sequences designated exons) and that B cell development is associated with molecular events resulting in splicing of *V, J*, and *C* gene segments. These studies have also demonstrated important intraspecies and interspecies differences in the number, order, and chromosomal location of κ- and λ-specifying genes.

Kappa. The organization and general features of human κ chain genes (located on chromosome 2) are similar to those found for the mouse κ chain genome.[20-24] In both species, beginning at the 5′ end of germline DNA, a short intron is followed by an exon coding for the hydrophobic 18-residue to 29-residue peptide or "extra piece"(XP) (Fig 3). Another short intron of ~100 nucleotide base pairs (bp) is followed by a *V* gene segment that specifies the synthesis of the remainder of the XP plus the first ~95 residues of the V_L. The *XP-*V_κ exons occur serially (in tandem), interspersed by introns of different lengths: *XP-V* exons encoding proteins closely homologous in sequence (ie, V region subgroups) are clustered in the DNA and are less widely separated than those encoding for proteins of other subgroups.[25,26] Human V_κ germline subgroup genes are also characterized by highly conserved intron segments far upstream (~4,500 bp) from the 5′ end of the *V* gene segment.[26] Multiple genes encoding

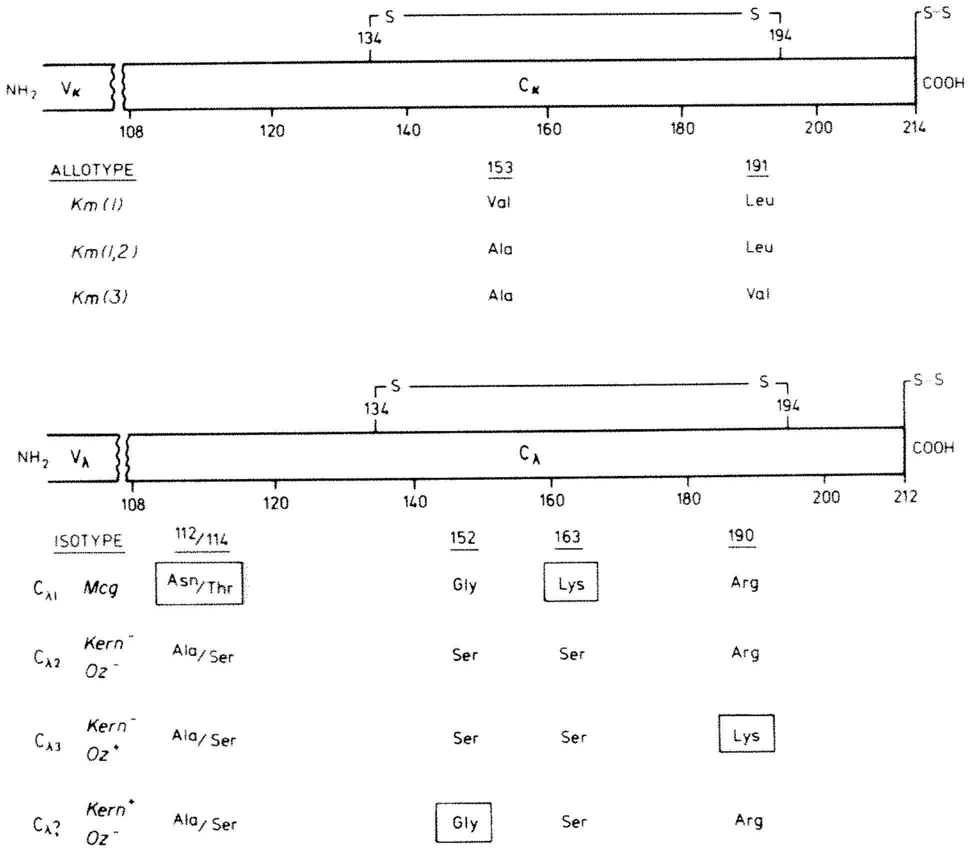

Fig 2. Localization of genetic factors on the constant region of human κ and λ light chains. The constant region of κ chains, C_κ, consists of 107 residues and is the product of a single gene of three allotypes, *Km(1), Km(1,2)*, and *Km(3)*, that are recognized serologically and defined chemically by the nature of the amino acid residues (as indicated) at positions 153 and 191. The constant region of λ chains, C_λ, consists of 105 residues and is the product of one multiple *C* gene segment. The $C_{\lambda 1}$, $C_{\lambda 2}$, and $C_{\lambda 3}$ genes encode for the isotypic factors *Mcg*, *Kern*$^-$Oz$^-$, and *Kern*$^-$*Oz*$^+$, respectively (although *Mcg*$^+$ proteins also bear the *Kern* marker, *Kern*$^+$*Oz*$^-$ proteins are products of yet another C_λ gene).[33] The amino acid residues characteristic for the *Mcg, Oz*, and *Kern* factors (positions 112/114/163, 190, and 152, respectively) are boxed; the alternative residues at these positions are unboxed. The residue numbering system is according to ref. 4.

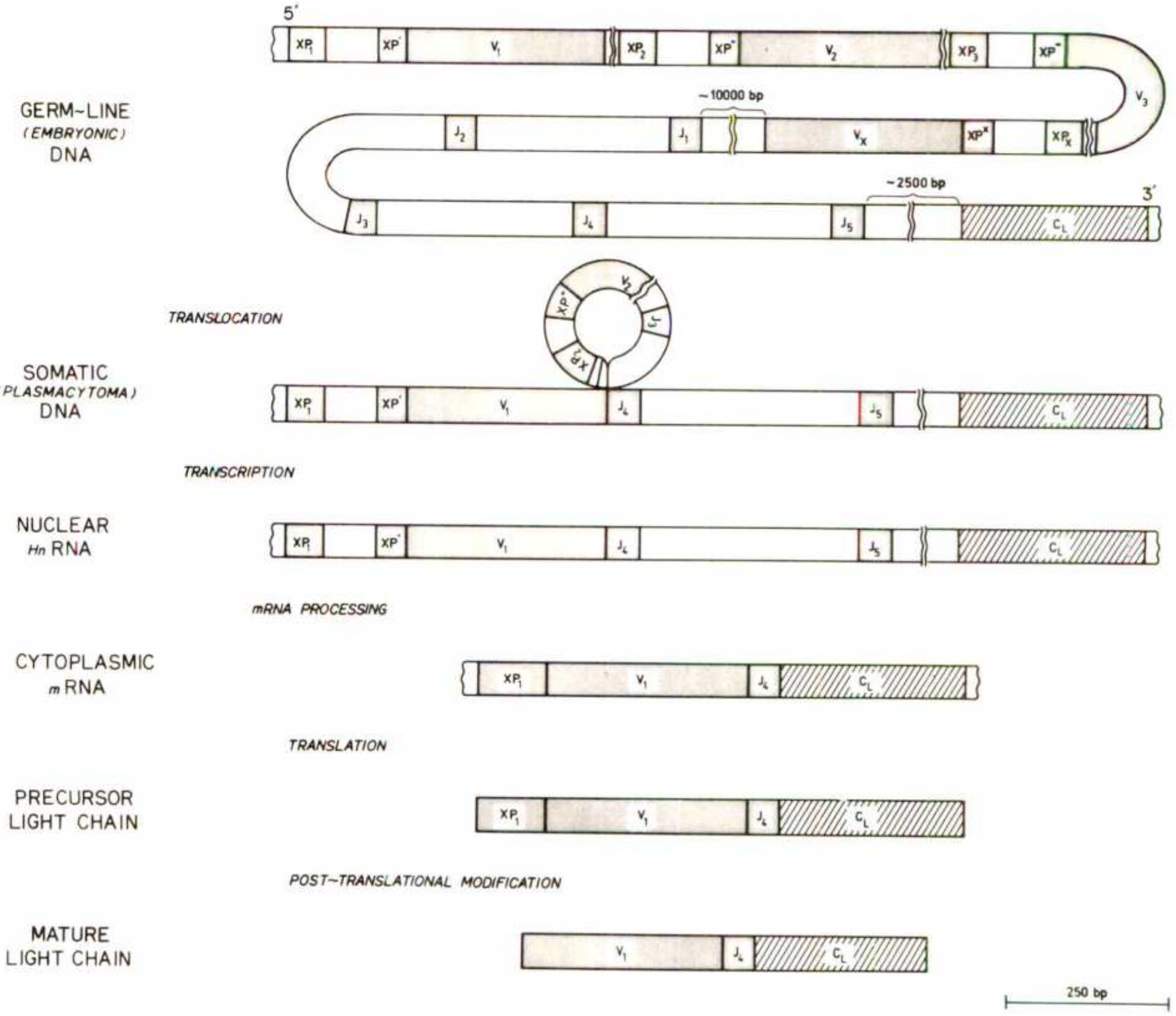

Fig 3. Schematic representation of the genetic events resulting in formation of a human immunoglobulin κ type light chain. The location, organization, and length on germline (embryonic) DNA of the gene segments (exons) encoding for the leader peptide or "extra piece" (XP), variable (V), and joining (J) segments of the variable (V_L) region and for the constant (C_L) region of a κ light chain are depicted (top). The multiple V_κ germline and associated *XP* gene segments are designated XP_1, XP'-V_1; XP_2, XP''-V_2; XP_3, XP'''-V_3; and XP_x, XP^x-V_x. A portion of the gene segment encoding for the XP, designated by the superscript notation, is joined or closely linked to the *V* segment. The five *J* encoding segments are shown as J_1, J_2, J_3, J_4, and J_5. The intervening, nontranslated nucleotide sequence (introns) segments are indicated by the clear spaces between each gene segment and, where known, the approximate nucleotide base pairs (bp) for each segment are given. Cellular differentiation is associated with translocation on somatic (plasmacytoma) DNA of specific germline DNA *XP*, *V*, and *J* segments, excision of intervening DNA nucleotides, and splicing together of the *V* and *J* segments. The *XP*, *V/J*, and the remaining *J* gene segments (plus the intergenic introns) are transcribed as high-mol-wt nuclear RNA (HnRNA). Processing of the HnRNA into the cytoplasmic mRNA to form the contiguous light chain gene involves excision of these nontranslated regions and the splicing together of the *XP*, *X-V/J*, and *C* gene segments. Posttranslationally, the precursor light chain product is converted into the native or mature form of light chain by intracellular proteolytic cleavage of the XP. A similar process results in the formation of a λ type light chain; however, there is not one but multiple C_λ gene segments, each apparently preceded by a *J* segment (ie, the J_λ segments are not arranged tandemly as in the κ genome).

for the major V_κ subgroups I, II, and III, and the single gene encoding for the minor V_κ subgroup IV have been sequenced.[27-33] The number of V_κ gene segments reflects the number of subgroups (and sub-subgroups) in the light chain repertoire; for humans, this is estimated to be ~30 to 50, based on protein and gene hybridization data. The recent identification of a novel V_κ gene[33] may signify a more extensive V_κ genome than is evidenced by expressed κ chains. A long intron (? 5 to 10,000 bp) separates the XP-V_κ segments from a series of five tandemly arranged *J* gene segments.[34] The *J* genes are interspersed by short introns, and encode the remaining ~13 residues of the V_L. A 1,250- to 4,000-bp intron separates the 3′ end of the last *J* segment from the 5′ end of the exon coding for a single V_κ region[35] which, in turn, is followed by another intron preceding the polyadenylate (poly A) at the 3′ terminating codon.

Lambda. Noncontiguous segments of germline DNA located on chromosome 22 also encode the XP, V, J, and C portions of the λ light chain.[20] The organization and association of λ chain-specifying genes are, however, strikingly different from those of κ genes, as evidenced chiefly from studies on mouse λ chains.[36] Mice have a limited number of V_λ genes (only two in certain inbred species): four groups, each preceded by one of the two V_λ genes. The limited number of V_λ genes, compared with V_κ, may account (in part) for the rarity in mice of λ light chains.

Less information is presently available on the human λ light chain genome. It differs from that of the mouse by having considerably more V_λ genes—a reasonable estimate is ~20 to 30, based on the serum Igκ/Igλ ratio and on the number of V_λ subgroups and sub-subgroups evidenced chemically and serologically (ref. 5 and A. Solomon, unpublished observations, 1986) and on cross-hybridization data with available gene probes. The sequences of V_λ genes encoding for proteins of the $\mathrm{V}_{\lambda\mathrm{I}}$, $\mathrm{V}_{\lambda\mathrm{II}}$, $\mathrm{V}_{\lambda\mathrm{III}}$, and $\mathrm{V}_{\lambda\mathrm{VI}}$ subgroups and of a J_λ gene have been determined through analyses of Igλ cDNA.[37-40] The presence in the human V_λ genome of other V_λ subgroups is evidenced by the finding of an unusual V_λ gene in a recombinant phage chromosome 22 library.[41] This gene encodes for a V region more homologous to mouse V_λ chains; a human λ chain bearing the V_λ sequence encoded by this gene has not yet been found. Although the number of human J_λ segments and their relationship to C_λ gene segments has not been established, based on nucleotide sequence data, it appears that there is one *J* segment per *C* segment (T. Vasechek and P. Leder, Howard University, personal communication, 1985). For the human C_λ genome, a preferential association of V_λ genes with J_λ-C_λ "clusters," as occurs in the mouse,[36] has not yet been demonstrated, but of possible significance is the recent finding that the J and C regions of two λVI light chains are identical to each other and to the J_λ and $C_{\lambda 3}$ gene segments of a rearranged $V_{\lambda\mathrm{VI}}$ gene.[41-43]

The complexity of the human C_λ genome *v* C_κ is evidenced by the number of C_λ isotypes identified chemically and serologically[10] and by the finding, in humans, of two distinct groups of C_λ genes.[44] For the first C_λ group, at least six C_λ genes, each apparently associated with its own J region, have been identified on chromosome 22, and additional polymorphism of the C_λ locus has been evidenced, including variations in gene numbers among normal individuals.[44,45] Three of these C_λ genes, $C_{\lambda 1}$, $C_{\lambda 2}$, and $C_{\lambda 3}$, have been sequenced. They encode for the *Mcg*, *Kern*$^-$, and *Kern*$^-$*Oz*$^+$ factors, respectively.[44] It is not known if any of the 11 other C_λ sequence differences noted by sequence analyses of monoclonal human λ chains[10] represent alleles of these three C_λ genes or are products of the $C_{\lambda 4}$, $C_{\lambda 5}$, or $C_{\lambda 6}$ genes (or of other C_λ genes). Less information is presently available on the localization of the second group of C_λ genes and its products, function, and relation to the first C_λ group. Most recently, the sequences of three genes from the second C_λ group have been reported. Two of these C_λ genes, *14.1* and *16.1*, have open reading frames and are each associated with their own *J* segment. The deduced amino acid sequences of the proteins encoded by these nonallelic genes are closely homologous but differ substantially (17 to 23 residues) from those encoded by the $C_{\lambda 1}$, $C_{\lambda 2}$, and $C_{\lambda 3}$ genes. The functional protein counterparts of the *14.1* and *16.1* genes have yet to be identified; the third C_λ gene lacks a *J* segment and presumably represents a pseudogene.

Evidently, the contribution of λ genes to antibody diversity has been accomplished in a different evolutionary process as compared to κ genes. Although κ genes use a tandem array of multiple V_κ and J_κ gene segments and a single C_κ gene, the λ genes not only have expanded *V* and *J* genes but also have multiple C_λ genes, thus forming an unexpectedly large family within the human genome.[46]

Formation of a complete light chain gene product. The transcription of genomic information encoding the amino acid sequence of the light polypeptide chain involves a well-ordered series of events that select and link together in somatic DNA the widely separated germline DNA exon segments encoding the XP, V, J, and C portions of the light chain (Fig 3). Although comparatively little is known about the selection process, the steps leading to the formation of a completely integrated light chain gene have been elucidated. This process occurs in B cells and is intimately associated with lymphocyte ontogeny. In somatic DNA, partial translocation of light chain gene segments occurs by the joining together of one *V* and one *J* segment to form a contiguous *V* gene; however, the *XP* to *V* and the *J* to *C* introns persist and are transcribed in the form of a high-mol-wt (27S) nuclear RNA (HnRNA).[47] The precursor HnRNA is processed to a "mature" cytoplasmic 13S mRNA species from which the message coding for the complete light polypeptide chain is translated. This RNA processing involves the excision of the *XP* to *V* and the *J* to *C* introns as well as the concomitant splicing together of the *XP-V/J-C* gene segments. Posttranslationally, the XP is cleaved enzymatically, which results in the "mature" or native form of light chain that is either associated with the heavy chain or secreted from cells as the Bence Jones protein. Although the function of the XP remains unknown, its hydrophobic residues may facilitate the binding of precursor chains to endoplasmic membranes.[48] The failure to cleave the leader (XP) peptide from the V_L may not allow newly synthesized protein to be released from ribosomes, thus resulting (in some cases) in a "nonsecretory" form of multiple myeloma.[48]

Generation of light chain V region (V_L) diversity. Recent information regarding the molecular events linking light chain synthesis and cellular differentiation has enhanced our understanding of the germline and somatic genetic mechanisms resulting in antibody sequence diversity. This information, although incomplete, accounts for the seemingly endless capacity of the immune system to form antibody molecules with different structures and specificities.[21-24]

The availability in germline DNA of multiple *V* and *J* gene segments capable of combinatorial joining provides an enormous potential for generation of V region sequence diversity. This diversity is limited only by the number of *V* segments (subgroup and sub-subgroup), *J* segments, and the variation that results from the combinatorial joining of a *V* and *J* gene segment.[49] Overlap in nucleotide base pairing at the V-J splice site, ie, the contribution of the 3′ end of the *V* gene and the 5′ end of the *J* gene to this site, generates new nucleotide coding sequences not specified in the untranslocated genes.[23] Position 96, coded for by the *J* segment itself or by the nucleotide base triplet resulting from *V-J* recombination, introduces further sequence variability in the third

CDR of the light chain. The tertiary structural implications of this residue have been previously discussed.

The number of *V* and *J* gene segments and the nucleotide diversity created by their combinatorial joining, however, cannot account for all the observed sequence differences among κ and λ chains. Mutational events affecting somatic DNA or RNA are evidenced from the discordance found between the amino acid sequence predicted from the nucleotide sequence of germline genes and the final sequence of their light chain products.[21-24,31,32,50] These mutations occur randomly in coding and flanking sequences, affect both the CDRs and FRs, and are seemingly limited only to the translocated (rearranged) *V-J* genes.[51,52] The functional importance of somatic mutation in generating antigen-binding specificity is not known. This mechanism may generate "useless" molecules or, more likely, serve to amplify the antibody repertoire; this remains to be determined.

Intragenic recombination of an error-prone repair system and gene conversion—an exchange of genetic information between chromosomes or on the same chromosome—also have been evoked as a mechanism in generation of antibody diversity.[53]

For the heavy chain V region, (V_H) sequence diversity in the junctional region between the V_H and J_H regions results from the presence of another gene segment that encodes for approximately seven amino acids in the CDR3 region and is designated the *D* (diversity) segment.[54] The presence of additional bases (termed *N* regions) at the junction of V_H/D and D/J_H translocated heavy chain gene segments also creates diversity in this region.[55] The light chain genome, however, lacks a *D* segment; the finding of extra bases at a V_L-J_L recombinant junction[56,57] suggests that *N*-like regions may occur in light chains.

Regulation of light chain gene expression. The precise mechanisms responsible for selecting particular *V* and *J* segments and for gene splicing and intron excision are not yet understood. Structurally homologous nucleotide sequences of regions flanking *V*, *J*, and *C* gene segments apparently serve as recognition signals for DNA ligases and polymerases.[22-24] For example, repeated and complementary inverted palindromic nucleotide sequences at the 3′ and 5′ ends of the *V* and *J* segments, respectively, can form a loop or stem structure that draws the segments together by an inversion (reciprocal exchange) process.[22,24,56,58] Substitutions or deletions of nucleotide recognition sequences essential for gene splicing can affect quantitatively the expression of light chains or render nonfunctional certain gene segments (pseudogenes).[24,33,36,58-63] Aberrant rearrangements of *V-J-C* gene segments can also result in protein fragments that lack genetically specified regions of the light polypeptide chain. For example, if *V-J* splicing results in the deletion of one nucleotide, the reading frame can shift so that a stop codon is generated in the *J* segment.

Combinatorial association of light chain gene segments is also facilitated by specific genomic factors (nucleotide sequences), known as promoters and enhancers, that are located in close proximity to the *V*, *J*, and *C* gene exons.[52,64-66] Germline and somatic light chain-encoding DNA also differ structurally in that gene translocation renders the nuclear chromatin DNase susceptible.[65] Other genomic regulatory mechanisms have been implicated in controlling the light chain gene expression, eg, regulatory genes or methylation of DNA bases. As will be discussed next, the deletion or unproductive rearrangement of κ genes may be a prerequisite for initiation of λ chain synthesis.

Hierarchy in light chain gene rearrangement. An explicit pattern of light chain gene rearrangement, κ preceding λ, has been evidenced through analysis of DNA derived from normal and neoplastic κ or λ chain-synthesizing B lymphocytes.[52,67-69] In κ chain-producing B cells, the $V_\kappa/J_\kappa/C_\kappa$ segments on one of the two κ alleles are first rearranged. If a functional gene fails to be formed, a rearrangement of the second κ allele results. In the meantime, the λ genes remain in germline configuration. In λ chain-producing B cells, the C_κ and J_κ (but not V_κ) genes are deleted or have undergone a nonproductive rearrangement. This seemingly orderly process of gene rearrangement is dependent on the formation of a functional light chain gene, and this event terminates the process at one of four stages: The configuration of the light chain genes in κ chain-expressing B cells is signified as κ^{+}/κ^{0}, λ^{0}/λ^{0}, or κ^{x}/κ^{+}, λ^{0}/λ^{0}; that of λ chain-expressing B cells is signified as κ^{x}/κ^{x}, λ^{+}/λ^{0}, or κ^{x}/κ^{x}, λ^{x}/λ^{+} (where the symbols + and *x* designate productive and nonproductive rearrangements, respectively, and *0* signifies germline configuration).[67] Exceptions to this hierarchical rearrangement ($\kappa \rightarrow \lambda$) have been found in several Burkitt lymphoma cell lines where rearranged λ chain genes are present in κ chain-expressing cells.[38,39]

The ability to determine Ig gene configuration in somatic cells has made it possible to classify, as of B or T cell origin, those leukemia or lymphoma cells lacking typical phenotypic features (the so-called "null" cells).[68] The detection of reorganized heavy (μ) or light chain genes in such cells signifies their B cell lineage (pre-B cell), and this information has prognostic and therapeutic importance, eg, those cases of chronic myelocytic leukemia in which, not infrequently, the blast cells in the acute phase of this disease ("blast crisis") are not myeloid but are of B cell origin.[70]

Isotype and allotype exclusion of light chain genes. Immunoglobulin molecules have either κ or λ light chains, and the genetic information encoding for both types is present in the genome of all normal individuals; thus, κ and λ chains represent isotypes. The fact that an Ig-synthesizing B cell expresses only one (κ or λ) of these two isotypes indicates the exclusion of the second member, an event referred to as isotypic exclusion.[24,52,64,65] A κ or λ light chain expressed by a B cell bears a single V region (subgroup or sub-subgroup) and J region isotype and, in the case of λ chains, a single C region isotype. This restriction results from a process in which gene segments are rearranged to form a functional gene product. Furthermore, in antibody-forming B cells, only one member of a chromosomal pair is expressed somatically—a phenomenon unique among autosomal genes and referred to as allelic exclusion.[24,52,64,65] Thus, one set of Ig genes is translocated and transcribed into a single functional gene on one chromosome, whereas the genes on the allelic chromosome remain in the germline (untranslocated) state. Misalignments in *V-J* recombination may also result in allelic exclusion by producing an untranslatable light chain gene.[33,39] Another mechanism evoked for control of gene expression and for allelic exclusion is the inhibitory effect that methylation of nucleotide bases has on Ig gene transcription.[65]

CONCLUSIONS

Knowledge of light chain molecular genetics has begun to make explicable the biological and chemical features of the immune system—the generation of the structural diversity necessary for antibody function, how Ig genes are regulated and expressed, and the relationship of these phenomena to normal and neoplastic B cell development. Studies at both the protein and gene level will continue to furnish new insights into these fundamental problems of human biology.

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PMN Heterogeneity: Long-Term Stability of Fluorescent Membrane Potential Responses to the Chemoattractant *N*-Formyl-Methionyl-Leucyl-Phenylalanine in Healthy Adults and Correlation With Respiratory Burst Activity

By Mark P. Fletcher and Bruce E. Seligmann

Polymorphonuclear neutrophils (PMNs) were isolated from 24 healthy adults 20 to 61 years of age and the proportion of cells that demonstrated depolarization responses to the synthetic chemotaxin *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) were enumerated using the lipophilic fluorescent cyanine dye 3,3′-di-pentyl-oxacarbocyanine [di-O-$C_{(5)}$(3)] and flow cytometry. The membrane potential responses were correlated to the cells' respiratory burst capabilities as measured by nitroblue tetrazolium (NBT) reduction and/or superoxide production in response to FMLP; 40.2% ± 15.1% (mean ± SD) of cells depolarized to FMLP. The mean SE for duplicate determinations 1 hour apart was 3.8% (range 0.4% to 13.6%, n = 15). There was no correlation between the percentage of depolarizing PMNs and the yield of cells, the percentage of immature cells, or the circulating WBC count. There was no difference in the average age of men (34.9 ± 9.9 years, n = 11) *v* women (33.8 ± 8.5, n = 13) (mean ± SD) studied, or in the percentage of depolarizing PMNs when men and women were compared (42.2 ± 10.6% *v* 43.1 ± 13.3%, respectively). However, there was a significant increase in the percentage of depolarizing PMNs with increasing age of women (r = .61, $P < .025$) but not men (r = .03, $P > .05$). Analysis of variance revealed significantly greater person-to-person variability in the membrane potential response than in day-to-day variability in the same person ($P < .0005$). The percentage of depolarizing PMNs in response to FMLP was significantly correlated with the percentage of NBT-positive cells from both purified PMNs and from whole blood (r = .849, $P < .0005$, r = .857, $P < .05$, respectively), and with the amount of superoxide produced, expressed as a percentage of that amount produced by cytochalasin B (cyto-B)-pretreated cells (r = .565, $P < .01$). The data indicate that PMNs from healthy adults demonstrate a heterogeneous membrane potential response to the chemotaxin FMLP that correlates with the cells' oxidative responsiveness and that intersubject differences can be detected. In addition, the proportion of responsive PMNs increases with increasing age in women.

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FUNCTIONAL heterogeneity of circulating human polymorphonuclear neutrophils (PMNs) has been appreciated for many years. Numerous assays that quantify individual cell responses, eg, nitroblue tetrazolium (NBT) test,[1,2] locomotion,[3-5] F_c rosette-positive cells,[6,7] have indicated that anywhere from 20% to 60% of morphologically indistinguishable PMNs in peripheral venous blood either are not responsive to varying stimuli or express surface receptor heterogeneity. Unfortunately, many assay systems that are used to identify and quantify PMN responses use cell suspensions and by design are able only to determine an averaged response of all cells present. Functional heterogeneity may in this way contribute to the high degree of variability in many in vitro functional assays that are presently used which employ PMN suspensions.

Technologic advances in flow cytometry have recently allowed the rapid evaluation of individual PMN membrane potential responses to various membrane active stimuli using indirect fluorescent probes of membrane potential.[2,8] These studies have indicated that normal human PMNs display heterogeneous membrane potential responses to the synthetic formyl peptide chemoattractant,[2,8] that this functional heterogeneity correlates with heterogeneous binding of the formyl peptides to specific membrane receptors,[8] and most recently, with the preferential binding of a monoclonal antibody directed at a human neutrophil surface structural determinant.[9] Although such data lend strong support to the concept of PMN heterogeneity, the origin of such structural and/or functional differences or their clinical significance has yet to be investigated.

In the present study, we determined the proportion of circulating PMNs from normal healthy adults that demonstrated depolarization responses to the synthetic chemotaxin *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), studying individuals over a 18- to 24-month period and correlating the proportion of depolarizing cells to the cells' respiratory burst capabilities. The data indicate that the proportion of depolarizing PMNs from a given subject in response to FMLP is stable enough over time in the absence of drug therapy or intercurrent illness to detect intersubject differences and that this proportion correlates with the proportion of oxidatively responsive cells.

MATERIALS AND METHODS

Subjects. Venous blood samples were obtained from healthy adult subjects 18 to 61 years of age, derived predominantly from a pool of University students and employees who had given informed consent. Subjects were asked to give blood at ~2- to 3-month intervals. At each sampling, the subjects were queried regarding recent or ongoing illness and ingestion of medications (especially nonsteroidal antiinflammatory drugs, antihistamine/decongestants, birth control pills, or megadose vitamin therapy), and were excluded from testing if any of these agents had been consumed in the past 24

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hours. Information as to the day of the menstrual cycle was not obtained.

Materials. Hanks' balanced salt solution (HBSS) or calcium and magnesium-free Hanks' (mHBSS), pH 7.4 (GIBCO Laboratories, Grand Island, NY), were used for all assays except as noted in the text. The croton oil derivative phorbol myristate acetate (PMA), cytochalasin B (cyto B), and the chemoattractant peptide FMLP (Sigma Chemical Co, St Louis, Mo) were dissolved in dimethyl sulfoxide (DMSO, Sigma) to make appropriate stock solutions which were further diluted such that the final concentration of DMSO in the reaction mixtures was ≤0.1% to 0.2% (vol/vol), a concentration found not to affect any of the assays performed or the viability of cells as measured by trypan blue exclusion or lactic dehydrogenase release. Obtained as follows were: ferricytochrome c (cyto c) (fraction VI from horse heart), superoxide dismutase (SOD, bovine liver), NBT (Sigma), Hypaque-M, 90% (Winthrop Laboratories, New York), Ficoll, and Dextran T250 (Pharmacia, Uppsala, Sweden). Dr Alan Waggoner of Carnegie Mellon University kindly provided the 3,3′ di-pentyl-oxacarbocyanine [di-O-$C_{(5)}$(3)], which was dissolved in ethanol and stored at 4 °C, and from which stock solutions were prepared by dilution into HBSS.

Leukocyte isolation. Peripheral blood PMNs from normal subjects were prepared by Ficoll-Hypaque gradient centrifugation followed by dextran sedimentation and hypotonic lysis as previously described.[10] This resulted in yields of 53.3% ± 2.0% (mean ± SEM, n = 40), and cell populations that routinely contained ≥91% neutrophils. All experiments were carried out within 4 hours of blood removal from the donor (2 to 3 hours after cell purification), since cells of greater out-of-the-body age begin to lose functional responsiveness, develop spontaneous depolarization, and demonstrate reduced proportions of depolarizing cells in response to FMLP.[2,9]

Flow cytometric assays of individual cell membrane potential responses. Flow cytometry (FC) was performed at 37 °C with a modified Ortho 50H Cytofluorograf (Ortho Diagnostics Systems, Inc, Westwood, Mass) equipped with an argon ion laser emitting 200 mW at 488 nm (Spectra Physics, Mountain View, Calif), and the data were analyzed with a 2150 Data Handling System as previously described.[11] In brief, forward light scatter (FS) and orthogonal (right angle) light scatter (90°-SC) were collected on all cells and allowed discrimination of viable from nonviable cells as well as the different WBC classes. Contaminating RBCs and debris (nonviable cells) and mononuclear cells as well as PMN aggregates could then be excluded from the fluorescence analysis by appropriate gating of the scatter parameters, and only the green fluorescence of those cells whose scatter parameters correspond to single viable PMNs could be observed.[11] Preliminary analyses of sorted PMN populations from both stimulated and resting cell preparations indicated that the electronically gated PMN population was 95% PMNs, 1% to 2% bands, 2% lymplocytes, and <1% eosinophils.

After isolation, the neutrophils were stored on ice in the absence of calcium or magnesium to minimize PMN self-aggregation as well as spontaneous secretion of granule contents and generation of superoxide anion. In preparation for assay, PMNs were suspended at 1.5 to 2.2 × 10^6 cells/mL in HBSS and incubated with the fluorescent probe, di-O-$C_{(5)}$(3) (12.5 nmol/L) at 37 °C with continuous stirring for 10 to 15 minutes to allow the cells to reach ionic and thermal equilibrium and demonstrate a stable fluorescence pattern.[2,11] On some days, this pattern was asymmetric, indicating prior activation or aging of the cells, but cell preparations with evidence of significant aging (ie, spontaneous depolarization) were not analyzed. Cells occasionally demonstrated a small degree of aggregation under these conditions, especially if the cells were older (at 4 hours), but when the cell concentration was kept <2.0 × 10^6/mL, spontaneous aggregation involved <5% of the cells. Preliminary experiments in which the cells were allowed to reach thermal equilibrium before addition of the probe indicated that the cells reached the same level of resting fluorescence and demonstrated fluorescence shifts upon activation identical to those that occurred when the probe was added before warming of the cells. Therefore, the di-O-$C_{(5)}$(3) was added before warming for convenience. The extent of cellular uptake of the fluorescent probe is dependent on the concentration of dye in the extracellular space as well as the relative potential difference across the membrane, so that small variations in unstimulated cells are seen as well as an age-related effect of spontaneous depolarization.[2,9] Although the absolute dye concentration is difficult to standardize, the proportion of depolarizing PMNs is independent of di-O-$C_{(5)}$(3) concentration in the range of 12.5 to 50 nmol/L, despite an absolute shift in fluorescence intensity of resting cells (data not shown). The activating stimuli used included the chemoattractant FMLP and the combination of 5 μg/mL cyto B (preincubation for 5 minutes) followed by FMLP. On certain occasions, the membrane perturbing agent and specific granule-selective secretagogue, phorbol myristate acetate (PMA), was used as a positive control to demonstrate cell responsiveness, because PMA produces a uniform loss of fluorescence by all cells.[2,11] Before and at timed intervals after addition of the stimulus, 20,000 PMNs were analyzed to demonstrate shifts in fluorescence intensity which reflected changes in membrane potential, and the data was stored on hard-disk memory for later analysis. Results are displayed as histograms of cell count *v* fluorescence intensity on a linear scale of 1 to 1,000, and the data are expressed as the percentage of cells that responded to the stimulus with a loss of fluorescence (depolarization). This percentage was determined at the time of maximum separation of the peaks after addition of FMLP and usually occurred by 1 to 1.5 minutes when the reaction was maintained at 37 °C. Previous work has indicated that depolarization is much more rapid than the repolarization response, which requires 10 to 20 minutes.[2] In most cases, the separation of the peaks was not complete; therefore, an estimation of the proportion of cells in each population was made by placing an electronic gate at the nadir between the two peaks and allowing the computer to determine the proportion of cells in each gated population. This method routinely yielded an estimate with a SD of <2% for multiple assessments of the same histogram.

Nitroblue tetrazolium (NBT) dye reduction test. NBT was dissolved in phosphate-buffered saline (PBS) (GIBCO) at 10 mg/mL by tumbling for 15 minutes at room temperature. This stock was used to dissolve FMLP ± cyto B to a final concentration of 2 × 10^6 mol/L and 10 μg/mL, respectively. PMNs in HBSS at 10 × 10^6/mL were prewarmed at 37 °C for 5 minutes in a thermostated stirred heating module (Pierce Chemical Company, Rockford, Ill), as were the NBT solutions containing the stimuli. The reaction was started by mixing equal volumes of cells and NBT solution and incubating further at 37 °C for 15 minutes. The cells were then cooled in an ice bath for 5 minutes and cytopreps were prepared, fixed in methanol, stained with 2% saffranin, and air dried. Two hundred PMNs were then scored for NBT positivity, using oil-immersion magnification, and the data were presented as the percentage of NBT-positive PMNs. In some assays, whole blood was used as the source of WBCs to determine the PMN responses to FMLP, but the assay and scoring were carried out as with the purified cells.

Assessment of O_2^- production in response to FMLP ± cyto B. Superoxide (O_2^-) production was assessed by measuring the SOD-inhibitable reduction of cyto c measured at 549 nm in a Lambda 3B UV/visible Dual-Beam Spectrophotometer (Perkin-Elmer) equipped with an automated and microprocessor-controlled 5 × 5 cuvette holder with thermoelectric temperature control and stirring capabilities linked to a model 3600 Data Station equipped with dual floppy-disk drives. Five individual reactions could be run simultaneously in disposal polyethylene cuvettes against their corresponding control reactions containing identical cell reaction components plus

20 μg SOD. Data are calculated as nmol of cyto c reduced per 10^6 PMNs per 15 minutes for FMLP ± cyto B-stimulated cells.

Statistical analysis. Where appropriate, means of experimental groups were compared using the *t* test for summary data, paired sample *t* test or analysis of variance using the components-of-variance model II.[12] Correlation coefficients were used to indicate the degree of association of experimental variables.

RESULTS

Subject selection. Twenty-four subjects have been studied, 14 on two or more occasions, over a period of 24 months to investigate the stability of their PMN membrane potential responses and correlate those responses with the cells' oxidative burst capabilities. Table 1 indicates the subject characteristics. We studied 11 men and 13 women with a mean age of 42.7 years (range 20 to 61 years). In general, the subjects were healthy and on no regular medications except occasional multiple vitamins. None were taking megadoses of vitamin C (ie ≥1 g/day). For the present studies, no subjects were studied if they had taken any antiinflammatory drugs within the prior 24 hours or were feeling ill.

Fluorescent membrane potential responses: Effects of sex, age, and intersubject differences. Figure 1 illustrates the fluorescent membrane potential responses of the PMNs of four subjects to stimulation with a saturating dose (10^{-6} mol/L) of FMLP. The cells of all four subjects were isolated and assayed on the same day, at the same time, and at the same age out-of-the-body. The percentages of depolarizing PMNs ranged from 28.3% to 59.4%, illustrating the variability of cell responsiveness between individuals on a given day. Although the baseline histograms of three of four of these cell preparations demonstrated mild skewing of the peak, indicating some baseline heterogeneity prior to stimulation, this was not commonly seen on those days on which only one donor was run; this baseline skew most likely represented a cell age effect,[2] since the stimultaneous preparation of the blood of four donors took additional time and therefore the cells were 3½ to 4 hours old at the time of analysis. Table 1 summarizes the membrane potential responses for the 24 subjects, indicating that 40.2% ± 15.1% (mean ± SD) of the PMNs depolarized in response to FMLP. To determine what factors were responsible for this variation in the percentage of depolarizing cells, we studied the reproducibility of one subject's response on a given day, the relationships of cell yield, donor sex, or donor age to the percentage of depolarizing cells, as well as the day-to-day *v* person-to-person variability [one way analysis of variance (ANOVA) using the components-of-variance model II[12]] of the membrane potential response. The mean SE for duplicate determinations on the same sample 1 hour apart was 3.8% (range 0.4% to 13.6%) (n = 15).

Figure 2 presents data on the relationship between cell yield (ie, the percentage of PMNs recovered from the whole

Table 1. Neutrophil Membrane Potential Responses to FMLP in 24 Healthy Subjects Studied Over 24 Months

Subject No.	Sex	Age (yr)	No. of Determinations*	Percentage (Mean ± SD)†
1	F	49	1	55.0
2	F	40	4	56.0 ± 4.8
3	F	28	2	43.5 ± 2.5
4	F	25	1	44.3
5	F	24	2	32.4 ± 0.4
6	F	35	3	68.9 ± 5.7
7	M	33	5	28.0 ± 6.0
8	M	61	2	41.2 ± 19.5
9	M	35	3	56.3 ± 10.3
10	F	25	3	15.8 ± 1.33
11	M	35	8	31.7 ± 10.1
12	F	20	1	33.0
13	F	43	2	49.1 ± 15.4
14	M	37	2	52.2 ± 1.9
15	M	35	1	45.3
16	M	25	2	47.5 ± 13.4
17	F	32	1	48.3
18	F	37	1	44.4
19	M	30	1	22.0
20	M	28	1	48.4
21	F	28	6	34.1 ± 10.9
22	F	41	2	36.4 ± 1.5
23	F	37	1	43.6
24	M	40	1	47.0
			n = 56	40.2 ± 15.1‡

Responses to *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) determined as described in legend to Fig 1.

*Number of times subject was studied over 24 months.

†Percentage of polymorphonuclear neutrophils losing fluorescence (depolarizing) after stimulation with FMLP.

‡Mean ± SD for all measurements on all subjects.

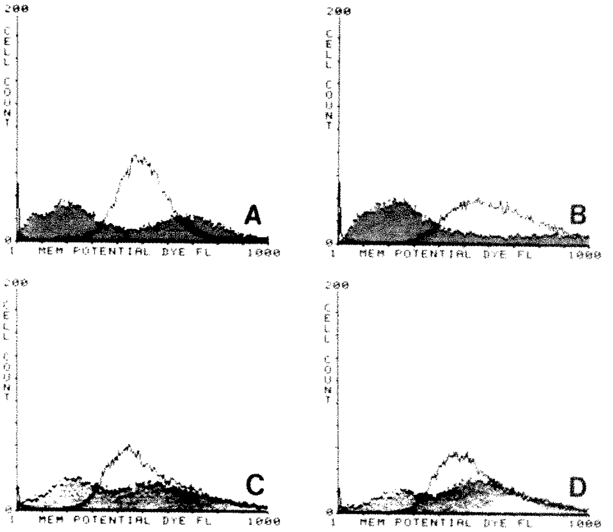

Fig 1. Heterogeneous membrane potential responses of polymorphonuclear neutrophils (PMNs) from different subjects. PMNs from four different subjects were isolated on the same day, using the same reagents, and were analyzed at the same age out-of-the body. Cells (1 to 2 × 10^6/mL) were equilibrated with the cyanine dye [di-O-$C_{(5)}$(3)] at 12.5 nmol/L final concentration for 10 to 15 minutes at 37 °C until a stable fluorescence pattern was seen (dark line). On addition at 10^{-6} mol/L of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), the cell preparation was reanalyzed after 1 minute to measure the change in the fluorescence histogram (shaded areas). Data are presented as the percentage of depolarizing cells (ie, those cells that have lost fluorescence). Green fluorescence is displayed as 1,000 linear channels. (A) Subject 2, 48.3%. (B) Subject 9, 59.4%. (C) Subject 11, 38.3%. (D) Subject 21, 28.3%.

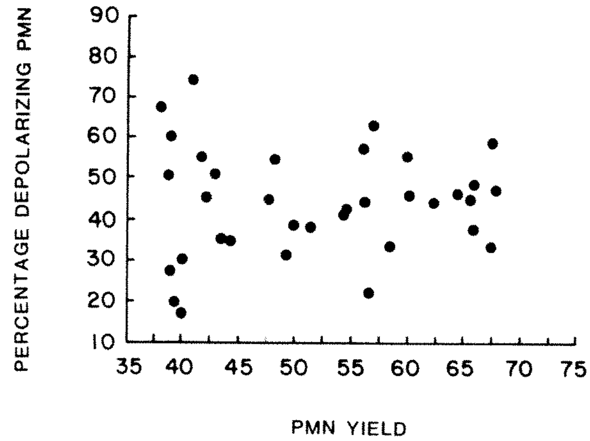

Fig 2. Lack of correlation between the membrane potential response and the yield of polymorphonuclear neutrophils (PMNs). Isolated PMNs were stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-8} mol/L) at 37 °C, and the percentage of depolarizing cells was measured and plotted against the cell yield.

blood sample) and functional response of the cells by plotting the percentage of depolarizing PMNs in response to FMLP against the cell yields, and indicates that no correlation was found; neither was there a correlation between the cell yield and the circulating WBC count or the percentage of immature neutrophils (data not shown). There was no difference in the average age of men (34.9 ± 9.9 years, n = 11) *v* women (33.8 ± 8.5 years, n = 13) (mean ± SD) or in the percentage of depolarizing PMNs when men and women were compared as a group (42.2% ± 10.6% *v* 43.1% ± 13.3%, respectively, mean ± SD). There was a significant correlation between the subjects' ages and their membrane potential response to FMLP, however. In Fig 3, a plot of the percentage of depolarizing cells *v* subject age demonstrates a significant correlation ($r = .35$, n = 24, $P < .05$), which was accounted for entirely by the women ($r = .61$, n = 13, $P < .025$) since there was no correlation between age and membrane potential responsiveness when men were analyzed as a group ($r = 0.03$, n = 11, $P > .05$). Thus, women but not men demonstrated an increased percentage of depolarizing PMNs with advancing age.

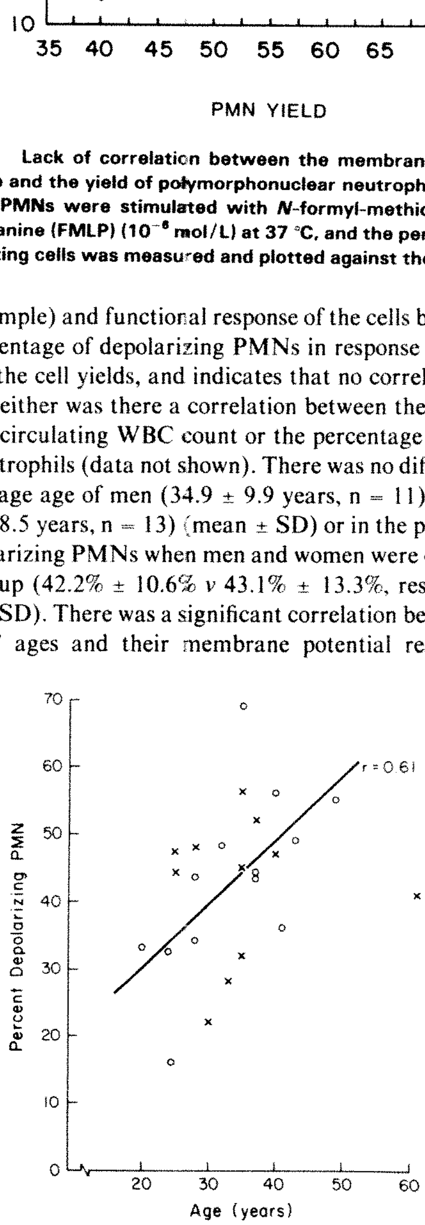

Fig 3. Correlation of the membrane potential response with subject age. Plot of the percentage of depolarizing polymorphonuclear neutrophils (PMNs) in response to 10^{-8} mol/L of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) *v* subject's age. Men (x) and women (o) are indicated separately. The solid line and corresponding *r* value reflects the correlation of membrane potential and female subjects only; there was no significant correlation for men as a group ($r = .03$).

The day-to-day *v* person-to-person variability of the membrane potential response was determined by performing a one-way ANOVA (Table 2). Data on the 14 subjects studied on two or more occasions indicate that the person-to-person variability was significantly greater than the day-to-day variability of a given individual and imply that the assay is capable of measuring true intersubject differences.

Correlation of membrane potential with NBT and superoxide responses. The relationship between the percentage of depolarizing PMNs in response to FMLP and the percentage of FMLP-elicited NBT-positive cells from the same subject is shown in Fig 4 and indicates a significant correlation between the two functional parameters ($r = .849$, $P < .0005$). The percentage of NBT-positive cells was 1½ to 2 times higher than that for the membrane potential response. We observed the formation of aggregates of 5 to 10 cells in the NBT-positive cell preparations after stimulation; this formation was not observed in the membrane potential assay, which had a lower cell concentration (1 to 2 × 10^6/mL). An attempt was made to count the NBT-positive cells regardless of their involvement in aggregates to reduce the bias toward counting only NBT-negative cells. Pretreating cells with cyto B prior to FMLP (which leads to the depolarization of 95% to 100% of the PMNs)[2,11] increased the NBT response to essentially 100%, confirming the previous observation that under appropriate conditions all the cells are functionally capable of responding to FMLP.[2,11] Similarly, stimulation with PMA led to a uniform depolarization by all cells in the membrane potential assay and a 100% response in the NBT test (data not shown).

The relationship between FMLP-elicited membrane potential changes and the amount of O_2^- produced as measured by cyto c is shown in Fig 5; again, a significant correlation is seen between the percentage of depolarizing PMNs and, in this case, the amont of O_2^- produced in

Table 2. Analysis of Variance: Comparing Intersubject *v* Intrasubject Variability of the Membrane Potential Response of PMNs from 14 Subjects Studied on Two or More Occasions

	Sum of Squares	Degrees of Freedom	Mean Square	*P* Value
Intersubject (within-groups variance)	8,308	13	639	
				$F = 8.11$
Intrasubject (pooled variance)	2,679	34	78.8	$P < .0005$

Data obtained from the 14 subjects in Table 1 who were studied on two or more occasions, using a components-of-variance model.[12] The *F* value of 8.11 indicates that significantly more variation exists between individuals than in the same individual studied on different days.

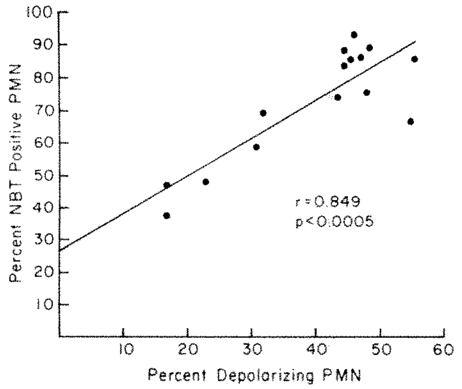

Fig 4. Correlation of the percentage of nitroblue tetrazolium (NBT)-positive polymorphonuclear neutrophils (PMNs) with the percentage of depolarizing cells in response to 10^{-6} mol/L of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). PMNs at 10 × 10^6 cells/mL and an NBT solution containing 2 × 10^{-6} FMLP were prewarmed at 37 °C, and equal volumes of each solution were mixed and further incubated for 15 minutes. The cells were briefly cooled on ice, and cytopreps were prepared on glass slides, fixed in methanol, and counterstained with 2% saffranin. Two hundred cells were counted, and NBT-positive cells (containing dark formazan deposits) were scored as percentage of positive cells. Percentages of depolarizing cells were obtained as in Fig 1.

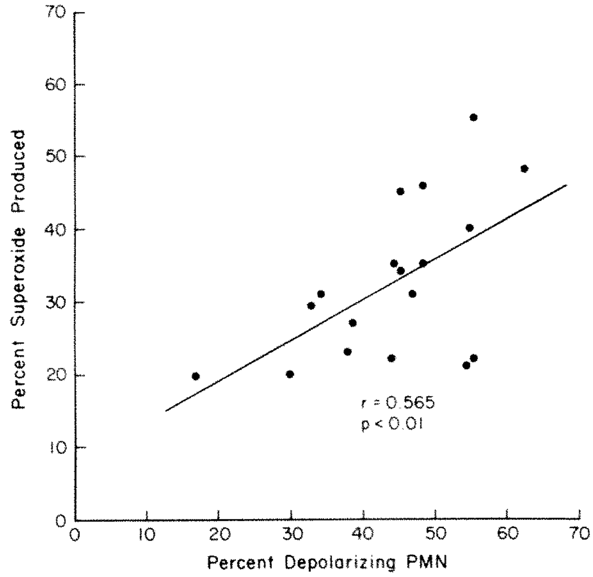

Fig 5. Correlation of superoxide (O_2^-) production with the percentage of depolarizing polymorphonuclear neutrophils (PMNs). PMNs (1.5 × 10^6/mL of Hanks balanced salt solution) were incubated with ferricytochrome c (cyto c) in a Perkin-Elmer lambda/3 dual-beam spectrophotometer with 5 × 5 cell holder, thermoelectric temperature control, and stirring capabilities for 5 to 10 minutes prior to the addition of 10^{-6} mol/L of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). The OD_{549} was recorded at 1-minute intervals against a reference cell that contained the same reactants plus 20 μg superoxide dismutase (SOD) In simultaneous reactions, the PMNs were pretreated with cytochalasin B (cyto B) (5 μg/mL) for 5 minutes prior to addition to FMLP. The OD_{549} at 15 minutes post-FMLP addition was used to calculate the nanomols of cyto C reduced per 10^6 cells; data are expressed as the percentage of superoxide produced by FMLP alone relative to that produced by cells pretreated with cyto B. The percentage of depolarizing PMNs was measured as in Fig 1.

response to FMLP alone (relative to that produced by cyto B + FMLP, reported as a percentage) (r = .565, $P < .01$). This correlation is not absolute, however, since the percentage of depolarizing PMNs may reach 60% to 70% in response to FMLP, whereas O_2^- production is never >40% to 50% of that seen with cyto B ± FMLP.

Correlation of the whole blood NBT test with that of purified PMNs and with membrane potential response. In selected studies, heparinized whole blood was stimulated with FMLP, and the PMN NBT response was quantitated. Hypaque-Ficoll–purified PMNs from the same sample were then tested for their responsiveness in the FMLP-elicited NBT and membrane potential response. Figure 6A plots the percentage of NBT-positive PMNs in whole blood against the percentage of NBT-positive Hypaque-Ficoll–purified PMNs; Fig 6B plots the whole blood NBT test against the percentage of depolarizing PMNs. The data demonstrate a significant correlation between the whole blood and purified NBT responses (r = .675, $P < .01$) and between the whole blood NBT and the membrane potential responsiveness of purified PMNs (r = .857, $P < .05$).

DISCUSSION

The concept of neutrophil heterogeneity has gained increasing support in recent years due to the considerable body of experimental data from unrelated assay systems indicating that both structurally and functionally defined

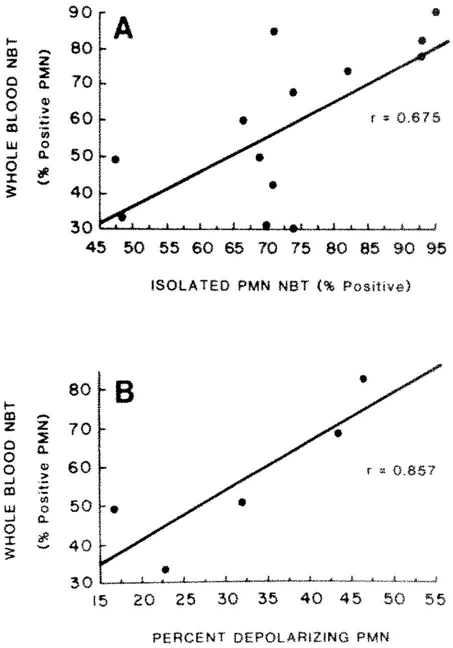

Fig 6. Correlation of the whole blood nitroblue-tetrazolium (NBT)-response of polymorphonuclear neutrophils (PMNs) to *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) with the NBT and membrane potential responses of subsequently purified cells. Heparinized whole blood was stimulated with 10^{-6} FMLP in the presence of NBT, and the proportion of NBT-positive PMNs was scored. Purified PMNs from the same sample were assessed for their NBT and membrane potential responses to FMLP; these responses were plotted against the corresponding whole blood NBT response (panels A and B, respectively).

subsets of mature neutrophils exist in the peripheral circulation. Beginning as early as the 1920s, investigators had observed great individual variability in the rate of PMN locomotion.[3] Since then, heterogeneity in locomotive activity,[4,5] phagocytosis,[13] oxidative metabolism,[1] protein synthesis,[14] and F_c receptor expression[6,7] have all been noted. More recently, the use of FC to demonstrate heterogeneity in membrane potential responsiveness of individual PMNs to *N*-formylated chemoattractant peptides.[2,11] and the concurrent demonstration of heterogeneous *N*-formylpeptide receptor expression[8] has indicated a link between the two. Most exciting is the very recent availability of monoclonal antibodies that recognize subsets of mature circulating PMNs.[9,15,16] These probes will aid in determining the role that PMN subsets may play in pathologic disease processes.

Although the existence of neutrophil heterogeneity seems well established, it remains unclear whether a single class of functionally more active PMNs is responsible for all the forms of heterogeneity determined to date, whether the heterogeneity reflects subpopulations derived from distinct stem cells, or whether heterogeneity represents different functional states of a single cell line.[13]

In the present studies, we have begun to address the role that the functional heterogeneity of PMNs may play clinically in relation to host-defense capabilities by investigating with FC the heterogeneity of FMLP-elicited membrane potential responses of peripheral blood neutrophils from healthy subjects. Our goal was to determine whether the proportion of depolarizing cells in a given individual was stable over time and whether intersubject differences could be consistently demonstrated. We believed that any attempts to study PMN subpopulations in patients with active inflammatory disease should be predicated on baseline data that established the reproducibility and stability of the membrane potential response in healthy subjects. We studied 24 subjects (14 on two or more occasions) over an 18- to 24-month period to determine their PMN membrane potential, NBT, and O_2^- responses to FMLP. The mean percentage of depolarizing PMNs for all subjects was 40.2 ± 15.1 (mean ± SD) and, although there was variation of the response in a given individual over time, an analysis of variance demonstrated significantly greater variation between individuals than between the same individual on different days, confirming and extending our previous observations[2,11] indicating that real intersubject differences can be detected with the FMLP-elicited membrane potential assay. The 40% response level is somewhat lower than the 65 ± 15.5 (mean ± SD) that we previously published.[2] However, the previous data were derived from a single determination on eight patients, whereas the present studies are the average of 57 determinations on 24 subjects and represent a larger data set with a range of values that significantly overlap the previous values. Unexpectedly, a significant positive correlation between the percentage of depolarizing PMNs and the age of the donor was noted, which was due entirely to the female subjects. Although no specific data were accumulated on the menstrual status of the women at the time of assay, it is interesting to speculate on the role of female sex hormones on the heterogeneous functional responses of PMNs, in lieu of previous studies indicating PMN functional defects in pregnant women[17] as well as in vitro evidence for inhibition of PMN respiratory burst activity by estrogen and progesterone compounds.[18] Studies are underway to assess the membrane potential and oxidant response of PMN during the menstrual cycle and to compare premenopausal and postmenopausal subjects as groups.

In an attempt to clarify further the nature of the intersubject differences noted in the present studies, the relationship between cell count, differential count, cell yield, or assay reproducibility to the membrane response was investigated. Although the average yield of PMNs was only 53% and therefore represents an analysis of only a proportion of the circulating cells, there was no correlation between the membrane potential response and the peripheral or differential WBC counts or the yield of granulocytes in the purified preparation (Fig 2). The variation in assay reproducibility on a given day was not large enough to explain the intersubject variations. Although reports have indicated that the functional responses of PMNs may be affected by cell aging[2] and cell isolation techniques,[19,20] the cells in the present studies were all isolated using the same procedure and reagents and often demonstrated intersubject differences when the PMNs of a number of subjects were studied on the same day (Fig 1). Such person-to-person variability seen on a given day cannot be totally explained by effects of cell aging or separation, although differential effects of these factors on subsets of cells may play a role in measured parameters of functional heterogeneity. Although the present data demonstrate intrinsic in vitro functional differences in PMNs after isolation by the present techniques, it is likely that this heterogeneity is a reflection of some true in vivo functional differences, since there is also a significant correlation with the functional responses of PMNs in whole blood (Fig 6). In addition, repeated study of the same individuals on multiple occasions indicates greater intersubject variation in membrane potential responsiveness than day-to-day variation in the same individual (Table 2). Thus, the intersubject differences likely reflect true differences in the proportion of FMLP-responsive PMNs that were circulating at the time the blood sample was taken. The concept of PMN functional heterogeneity is further supported by demonstrations of antigenic heterogeneity by a number of labs using independently obtained PMN-monoclonal antibodies.[9,15,16]

In addition to the heterogeneous membrane potential response, PMNs in the present studies also demonstrated heterogeneous oxidative responses to FMLP as measured by scoring individual cells for NBT reactivity and by measuring superoxide production in response to FMLP relative to that in response to Cyto B + FMLP. Figures 4 through 6 indicate that good correlations exist between the percentage of depolarizing PMNs and the oxidative responses of purified cells as well as with the less manipulated cells in whole blood. It has been previously demonstrated by a number of laboratories that pretreating PMNs with cyto B enhances the subsequent responsiveness of PMNs to FMLP in both superoxide[21] and secretion assays.[22] Using FC, we previously showed that pretreating PMNs with cyto B recruited essentially 100% of the cells to depolarize in response to FMLP.[2,11] If the increase in measured superoxide production with cyto B pretreatment is due to the recruitment of previously FMLP-unresponsive PMNs, the ratio of O_2^- produced by FMLP alone to that produced by cyto B + FMLP should correlate with the percentage of FMLP-responsive cells in the membrane potential assay. Figure 5 shows such a correlation and lends further support to the concept that FMLP-mediated membrane potential responses are associated with FMLP-mediated respiratory burst activities,[23] although the correlation is not absolute and may reflect the fact that in addition to recruiting more cells to respond, cyto B probably enhances the measured oxidant production of each cell to FMLP.

These data suggest that the subpopulation of PMNs that depolarizes to FMLP represents a functionally more responsive group of cells that may be of primary importance in early host defense mechanisms involving PMNs at inflammatory sites. Preliminary data indicate that the proportion of depolarizing PMNs from patients with ongoing inflammatory processes may be enhanced when compared with control subjects' cells studied on the same day (M.P. Fletcher, and Bruce E. Seligmann, unpublished observations). One may question whether a given individual's ability or lack thereof to recruit more circulating PMNs into the "depolarizing" or functionally responsive subpopulation during an infectious or other inflammatory insult has any bearing on a successful host defense mechanism. Reports of increased proportions of NBT-positive PMNs in subjects with bacterial and inflammatory diseases[24,25] support this concept. In addition, it has been recently demonstrated that leukotriene B_4 is capable of acting as a "priming agent" in a manner similar to that of cyto B by recruiting unresponsive PMNs into a more functionally responsive state.[26] Such recruitment by LTB_4 is consistent with the data of Gay and co-workers who have demonstrated enhancement of FMLP-induced O_2^- production by LTB_4 in a concentration-dependent fashion,[27] and adds more evidence in support of the importance of PMN functional heterogeneity.

The present studies indicate that purified PMNs from healthy subjects demonstrate a heterogeneous membrane potential response to the synthetic chemotaxin, FMLP, which correlates with the oxidative responses of these as well as with those of the unpurified PMNs in the whole blood cells, and which appears to be relatively stable in a given individual over time. Understanding the origin and importance of neutrophil heterogeneity in normal host defense mechanisms is in its infancy, but with the availability of fluorescent membrane potential-sensitive dyes used in conjunction with the technology of FC, correlated measurement of structural and functional parameters of individual cells is facilitated; thus, the significance of such heterogeneity is likely to be clarified in the near future.

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Cell-Mediated Suppression of Megakaryocytopoiesis in Acquired Amegakaryocytic Thrombocytopenic Purpura

By Alan M. Gewirtz, Michelle Keefer Sacchetti, Rebecca Bien, and William E. Barry

Acquired amegakaryocytic thrombocytopenic purpura (AATP) is a disorder of hematopoiesis characterized by severe thrombocytopenia due to a selective reduction or total absence of megakaryocytes in an otherwise normal-appearing bone marrow. Although the development of autoantibodies directed against cells in the megakaryocyte progenitor cell pool has been implicated in the pathogenesis of this disorder, cell-mediated suppression of megakaryocytopoiesis has not been described. Accordingly, we report two cases of AATP in which in vitro suppression of megakaryocyte colony formation by autologous ancillary marrow cells was demonstrable. Light-density bone marrow mononuclear cells (MNCs) obtained from both patients were either plated directly into plasma clot cultures, or after first being depleted by adherent monocytes (MØ) or T lymphocytes using standard methodologies. In some experiments, the depleted ancillary marrow cells were recovered for autologous co-culture studies with the MNCs from which they had been depleted. Megakaryocyte colony formation was detected in the cultures using an indirect immunofluorescence assay with a rabbit anti-human platelet glycoprotein antiserum. Removal of MØ (n = 6), or T lymphocytes (n = 4) from normal marrow MNCs had no apparent effect on colony formation. In contrast, depleting T lymphocytes from the MNCs of patient 1 significantly augmented megakaryocyte colony formation; a similar effect was observed after depleting MØ from the MNCs of patient 2. This observed augmentation in colony formation could be abrogated by autologous co-culture with the putative suppressor cell at effector cell/target cell ratios of 1:10 in the case of T lymphocytes or 1:5 in the case of MØ. Neither suppression nor stimulation of megakaryocyte colony formation was observed after culturing normal MNCs with autologous T cells (n = 4) or MØ (n = 3) at similar or greater ratios. We also observed inhibition of megakaryocyte colony formation after culturing normal MNCs in the presence of tissue culture medium conditioned by the MØ of patient 2. This effect was shown to be specific for megakaryocytes since this same conditioned medium had no significant effect on BFU-E and CFU-E–derived colony formation by autologous marrow mononuclear cells. These results suggest that: (a) both T cells and MØ are capable of exerting a regulatory effect on the proliferation of human megakaryocyte progenitor cells (CFU-Meg); (b) in the case of MØ, a soluble factor elaborated by these cells may be responsible for suppressing CFU-Meg growth; and (c) aberrant ancillary cell–megakaryocyte progenitor cell interactions may lead to clinically significant disease.

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ACQUIRED amegakaryocytic thrombocytopenic purpura (AATP) is a disorder of hematopoiesis characterized by severe thrombocytopenia due to a selective reduction or total absence of megakaryocytes in an otherwise normal-appearing bone marrow.[1,2] A number of factors have been implicated in the pathogenesis of this condition. AATP may be an early manifestation of an intrinsic stem cell defect since it has been documented to be a prodrome of acute leukemia and aplastic anemia.[1,2] Alternatively, AATP may result from selective damage to the megakaryocyte progenitor cell compartment after exposure to certain infectious agents,[3,4] drugs,[5] or toxins.[6]

Recently, Hoffman and co-workers described two cases of AATP that were associated with the development of a complement-fixing autoantibody directed against an early megakaryocyte progenitor, or the megakaryocyte colony-forming unit (CFU-Meg) itself.[2,7] To our knowledge, an association between cell-mediated suppression of in vitro megakaryocytopoiesis and impaired bone marrow megakaryocyte production has not been described. We report studies on two patients with AATP which suggest that bone marrow T lymphocytes and adherent cells of the monocyte-macrophage lineage may both suppress megakaryocytopoiesis in vitro. Suppression occurred at physiologically relevant target cell/suppressor cell ratios and, in the case of adherent cell-mediated inhibition, appeared to be caused by a soluble factor elaborated into the cell's environment. Our data suggest that the in vitro phenomena we observed may have had in vivo significance, and that some cases of AATP may be the result of cell-mediated immunologic injury of megakaryocyte progenitor cells.

MATERIALS AND METHODS

Case Reports

Patient 1. A 58-year-old woman who was seen by her physician had a 6-week history of easy bruising and spontaneous ecchymosis formation. These complaints were temporally associated with the administration of erythromycin for periodontal disease but did not abate when the drug was discontinued. Physical examination revealed only multiple skin ecchymoses. The spleen was not enlarged. A complete blood count disclosed a platelet count of 22,000/μL with no other abnormality. Blood chemistries, a serum protein electrophoresis, folic acid and B_{12} levels, an ANA, and a nuclear scan of the spleen were normal or negative. A bone marrow aspirate and biopsy disclosed a marked reduction in bone marrow

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megakaryocytes. Marrow cellularity was otherwise normal, maturation was orderly, and iron stores were adequate. Platelet survival, performed as previously described,[1] was within normal limits. A diagnosis of AATP was made

Prednisone therapy (60 mg/day) was administered for 14 days, during which time the patient's platelet count remained <20,000/μL. The drug was therefore discontinued, and the patient was referred to Temple University Hospital for bone marrow culture studies. At the time of study, her platelet count was 44,000/μL, her WBC count was 5,700/μL, and her hematocrit was 37.3% with a mean corpuscular volume (MCV) of 93 fL. A repeat marrow aspirate confirmed the presence of only rare megakaryocytes. Cellularity and maturation were otherwise normal.

Three weeks later, the patient was found to have an impacted wisdom tooth. Erythromycin (2 g/day) was reinstituted and continued for 16 days without reduction in her platelet count (~51,000/μL). Her condition did not improve, and she was advised to undergo an extraction. Her preoperative platelet count was 72,000/μL. The patient tolerated the procedure well without platelet infusions. Postdischarge, her platelet count continued to increase. One month later, repeat bone marrow culture studies were performed when her platelet count was 92,000/μL and rising. Six months after her initial presentation, the platelet count was normal (192,000/μL). The patient remains entirely well.

Patient 2. A previously well 67-year-old housewife had a 4-month history of easy bruising. Her physical examination was normal except for the presence of petechiae and scattered ecchymoses. Initial laboratory studies disclosed a platelet count of 13,000/μL. The WBC count was 13,500/μL with a normal differential. The hematocrit was 45% with a MCV of 94 fL. A reticulocyte count was 2.1%. An ANA was negative. A bone marrow aspirate and biopsy were both hypercellular with normal erythroid and granulocytic maturation. Megakaryocytes were nearly absent in each. Cytogenetic analysis of the bone marrow cells disclosed a normal female karyotype. A diagnosis of AATP was made.

Attempts were made to raise the platelet count, first with prednisone (60 mg/day for 30 days) and then with colchicine (0.6 mg/day for 60 days). Each drug was associated with a transient rise in the platelet count (80 × 10³/μL and 220 × 10³/μL maximum, respectively), lasting only a few weeks. No further therapeutic interventions were attempted. Twenty-four months after diagnosis, the patient maintains a platelet count which ranges between 10,000 and 50,000/μL, but she has had no serious bleeding episodes.

Methods

Megakaryocyte progenitor cell assay. Informed consent was obtained prior to aspirating bone marrow from patients and normal volunteers. Aspirated bone marrow was diluted 1:1 in α-medium without nucleosides containing 20 U/mL of preservative-free sodium heparin. A single cell suspension was prepared, layered, over an equal volume of Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) (sp gr 1.077 g/mL) and then centrifuged for 25 minutes at 1,500 rpm at 4 °C. The LDMNCs were collected and washed and then cultured in plasma clot cultures at a concentration of 5 × 10⁵ cells/mL as previously described.[8,9] All cultures, unless otherwise indicated, were supplemented with normal human AB serum to a final concentration of 30% (vol/vol) to provide essential growth factors. In one series of experiments (Table 1), cells were also cultured in 30% autologous serum or in 20% AB serum + 10% aplastic anemia serum, a known source of MEG-CSA.[10,11]

Megakaryocyte colonies were enumerated as previously described using a highly specific rabbit anti-human platelet glycoprotein (anti-PGP) antiserum in an indirect immunofluorescence assay.[8] A cluster of three or more fluorescent cells was scored as one megakaryocyte colony. Unless otherwise stated, all data are reported as the mean ± SEM of megakaryocyte colonies counted in quadruplicate culture dishes.

Table 1. Initial Assessment of Patients' Megakaryocyte Progenitor Cell Cloning Efficiency

Culture Condition	Megakaryocyte colonies/5 × 10⁵ Cells* Patient 1	Patient 2	Control†
Normal AB serum	0.75 ± 0.5	25 ± 10	36 ± 5
AB serum + aplastic anemia serum	0.50 ± 0.4	109 ± 31	57 ± 7
Autologous serum	2.0 ± 1.6‡	292 ± 53	NT

NT, not tested.

*Results expressed as mean ± SEM of quadruplicate cultures.

†Aggregate results from studies of 11 normal donors.

‡Mean ± SEM of duplicate cultures.

Erythroid progenitor cell assays. CFU-E and BFU-E were harvested, stained, and scored as previously described.[2] The concentration of erythropoietin (Connaught, Step III, Sweetwater, Pa) in the cultures was 2 IU/mL.

Bone marrow smear preparation. Marrow smears were prepared at the time of initial presentation from aspirated marrow as previously described.[2,8] In brief, a small aliquot of marrow was collected into EDTA and smeared directly onto clean glass slides. Specimens to be immunochemically examined for megakaryocytes were air dried, fixed in methanol/acetone (1:9), and then stored frozen until probed with anti-PGP antiserum[8] for the presence of both morphologically and nonmorphologically recognizable megakaryocytes.

Cell separation and recovery procedures. Bone marrow mononuclear cells (MNCs) obtained as described above were depleted of monocyte-macrophages (MØ) by adherence to serum-coated plastic Petri dishes using the method of Kumagi and co-workers.[12] After being charged with the MNC cell suspension, (5 mL at 1 × 10⁷ MNCs/mL) dishes were incubated at 37 °C in humidified air containing 5% CO_2 for 2 hours. The nonadherent cells were then removed from the culture dishes by gentle rinsing with fresh α-medium, washed once again in α-medium, and then resuspended for further culture studies.

Adherent cells were recovered for co-culture experiments by adding 0.2% EDTA in phosphate-buffered saline (PBS) to the dishes and placing them at 4 °C for 30 minutes.[12] Adherent cell-depleted mononuclear cells contained ≤5% contaminating nonspecific esterase positive cells.[13] Cells adhering to the dishes were ≥90% nonspecific esterase staining positive.

T lymphocytes (T cells) were removed from the MNCs by rosetting with neuraminidase-treated sheep RBCs.[14] Rosetted cells were separated from nonrosetted cells by a second Ficoll-Paque density gradient sedimentation. Nonrosetted MNCs were designated T lymphocyte-depleted and contained <5% T cells as assessed by immunofluorescence staining with OKT11 monoclonal antibody (Ortho, Raritan, NJ). Mononuclear cells in the RBC pellet were T lymphocyte enriched, as demonstrated by fluorescence staining with OKT11 (≥95% positive). They were recovered for autologous co-culture studies by hypotonic lysis of the sheep RBCs with ammonium chloride (0.83% in Tris buffer).

To calculate the expected number of megakaryocyte colonies after cell depletion procedures, the following formula[15] was used: Expected colonies (in T cell or MØ-depleted MNCs) equals (Meg colonies/5 × 10⁵ unseparated MNCs) times 100 divided by 100-[%T or MØ cells in control MNC)-(% T or MØ cells in depleted MNC)].

Preparation of patient and normal control adherent cell conditioned medium. Marrow mononuclear cells (~1 × 10^6) remaining attached to the serum-coated plastic Petri dishes after five rinses with PBS were overlaid with 5 mL of α-medium containing 10% human AB serum. The dishes were then incubated for 7 days at 37 °C in an atmosphere containing 5% CO_2 at 95% humidity. At the end of that time, the medium was aspirated from the dishes, clarified by centrifugation (10,000 g for 5 minutes), and frozen at −80 °C until needed.

Statistics. Statistical significance of data comparisons was tested using an unpaired Student's *t* test.

RESULTS

To determine where the block in megakaryocyte maturation existed in these patients,[2,6] marrow smears prepared at the time of their initial presentation were stained with anti-PGP antiserum. In patient 1, a rare fluorescein-positive cell, which under a phase-contrast observation could be morphologically identified as a mature megakaryocyte, was observed in the available study material. In patient 2, no fluorescent cells of any kind were observed. These results suggested that the perturbation in megakaryocytopoiesis in these patients existed at the progenitor cell level.

Studies were then carried out that were designed to detect either intrinsic defects in the patients' megakaryocyte progenitor cells or the presence of serum inhibitors that could have been suppressing their growth. Intrinsic defects were sought by assaying the patients' marrow mononuclear cell fraction for residual CFU-Meg cloning efficiency in response to an exogenous source of Meg-CSA in the form of aplastic anemia serum. The patients' serum was screened for humoral inhibitors by substituting fresh, autologous serum (not heat inactivated) for normal AB serum in some marrow cell cultures. The total human serum concentration (vol/vol) in all cultures was equivalent. Results obtained, along with reference data accrued from 11 normal control subjects, are shown in Table 1. When these experiments were carried out, patients 1 and 2 had platelet counts of 44,000 and 10,000, respectively, and only rare megakaryocytes in their marrow aspirates.

Patient 1 demonstrated markedly abnormal megakaryocyte colony formation in normal AB serum, autologous serum, and in the presence of aplastic anemia serum (Table 1). Her CFU-Megs were therefore either severely damaged or their development was being suppressed by as yet undefined influences.

Residual Meg-CSA responsive CFU-Megs were detected in patient 2. Furthermore, the addition of fresh autologous serum to this patient's cell cultures resulted in an 11.7-fold increase ($P < .001$) in megakaryocyte colony formation as compared with growth observed in AB serum alone and a 2.7-fold increase ($P < .05$) as compared with colony formation observed in the presence of aplastic anemia serum (Table 1). These data tended to exclude the presence of a serum inhibitor of CFU-Meg but failed to provide a reason for the aborted production of megakaryocytes in vivo.

We therefore sought to determine whether bone marrow T lymphocytes or adherent monocyte-macrophages were suppressing the patients' megakaryocytopoiesis by selectively depeleting these cell types from the patients' marrow cells prior to cloning.

Depletion of adherent cells from six normal control marrows and from the marrow of patient 1 had no statistically significant effect ($P > .05$) on megakaryocyte colony formation (Fig 1A). In contrast, the adherent cell-depleted marrow cells of patient 2 gave rise to 121 ± 44 megakaryocyte colonies *v* 25 ± 10 from unseparated marrow cells (Fig 1A). As adherent cells comprised only 12% of the unseparated marrow mononuclear cells of patient 2, we would have expected a maximum of 26 colonies to form as a result of progenitor cell concentration due to the cell depletion procedure. Sixty days later, when her platelet count was 8,000/μL and her marrow composition was unchanged, these studies were repeated and similar results were obtained. At that time unseparated marrow cells formed only 1.0 ± .48 megakaryocyte colonies/5 × 10^5 mononuclear cells cloned, but depletion of adherent cells led to the formation of 8 ± 1 megakaryocyte colonies ($P < .001$).

These studies suggested that an adherent cell may have been suppressing megakaryocytopoiesis in patient 2. To test this hypothesis, the adherent marrow cells of patient 2 and

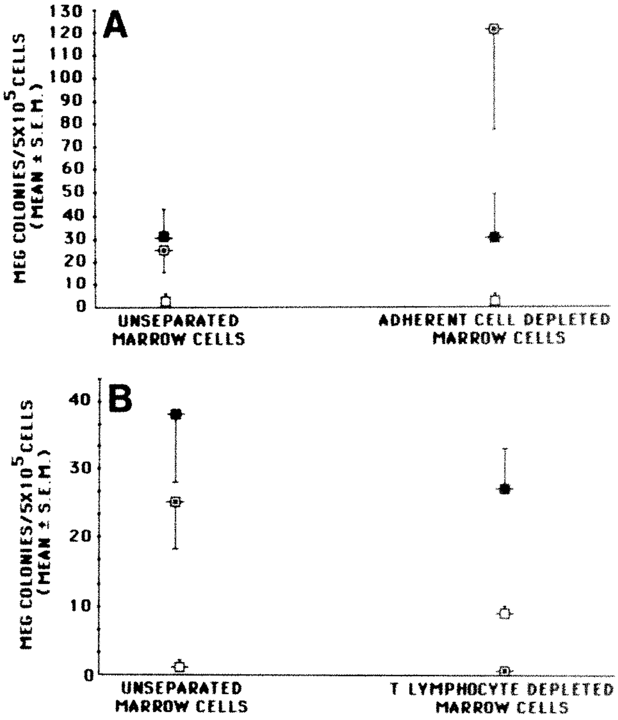

Fig 1. Effect of ancillary marrow cell depletion on patients' megakaryocyte progenitor cell cloning efficiency. At the time of study, patients 1 and 2 had platelet counts of 44,000/μL and 10,000/μL, respectively. Marrow megakaryocytes were rare in each. Serum used to supplement the cultures was derived solely by normal AB donors. Panel A shows the effect of adherent cell removal. Aggregate results of adherent cell depletion from six normal controls is shown for comparison purposes. Panel B shows the effect of T lymphocyte depletion with aggregate results from four normal control marrows for comparison. Results are expressed as mean ± SEM of colonies enumerated in quadruplicate plates. ■, control; □, patient #1; ▣, patient #2.

Table 2. Co-Culture Experiments With Autologous Adherent Cells

Adherent Cell/Target Cell* Ratio	No. of Colonies Formed	Effect on Megakaryocyte Colony Formation (%)
Patient 2		
Target cells alone	8.0 ± 1.0	—
1:10	8.0 ± 0.7	↔ 0
1:5	0.8 ± 0.5	↓ 91†
1:2	1.0 ± 0.4	↓ 88†
1:1	0.0 ± 0.0	↓100†
Controls‡		
Target cells alone	31 ± 14	—
1:10	29 ± 9	↓ 6§
1:5	28 ± 6	↓ 10§
1:2	23 ± 8	↓ 26§
1:1	NT	—

NT, not tested.

*Adherent cell-depleted marrow mononuclear cells.

†$P < .001$ (all cultures performed in quadruplicate).

‡Aggregate data from three normal individuals.

§$P > .05$.

adherent marrow cells from three normal controls were recovered for autologous co-culture studies with the marrow mononuclear cells from which they had been removed (Table 2). At adherent cell/target cell ratios of 1:10, 1:5, and 1:2, megakaryocyte colony formation was statistically unaffected in the normal controls. In contrast, colony formation by the cells of patient 2 was inhibited ≥90% ($P < .001$) at effector/target cell ratios of 1:5 and above. A soluble factor elaborated by the patient's adherent cells appeared to be responsible for this effect, since addition of an adherent cell-conditioned medium (ACCM) prepared from the patient's marrow MØ significantly inhibited megakaryocyte colony formation by autologous marrow mononuclear cells (Fig 2, panel A). On the day this experiment was performed, the patient's mononuclear cells gave rise to 63 ± 8 megakaryocyte colonies per 5×10^5 cells plated. In the presence of 5% and 10% patient ACCM, this number was reduced to 16 ± 0.5 ($P < .02$) and 7 ± 3 ($P < .01$), respectively. A control ACCM assayed simultaneously against the same target cells had no statistically significant effect ($P > .05$) on megakaryocyte colony formation (Fig 2, panel A). The inhibitory activity was abolished by heating the ACCM for 30 minutes at 60 °C (Fig 2, panel B).

The ACCM of patient 2 also inhibited megakaryocyte colony formation by three of the four normal marrow mononuclear cells against which it was tested (Fig 2, panel C). In the experiments in which this effect was observed, inhibition averaged 74% in comparison to baseline colony formation. This result was in marked contrast to results we obtained in the past with ACCM derived from normal cells, which had no apparent effect on CFU-Meg cloning efficiency.[16] A control ACCM assayed simultaneously with the ACCM of patient 2 in the fourth experiment also had no significant effect on megakaryocyte colony formation (baseline colony formation, 117 ± 11 [mean ± SEM/5×10^5 cells plated]; in 5% control ACCM, 114 ± 6; in 10% control ACCM, 129 ± 26).

We also sought to determine the relative specificity of this soluble inhibitor for cells of the megakaryocyte lineage by assaying CFU-E–derived and BFU-E–derived colony formation in the presence of the autologous or normal control ACCM on two separate occasions (Fig 3). Final concentra-

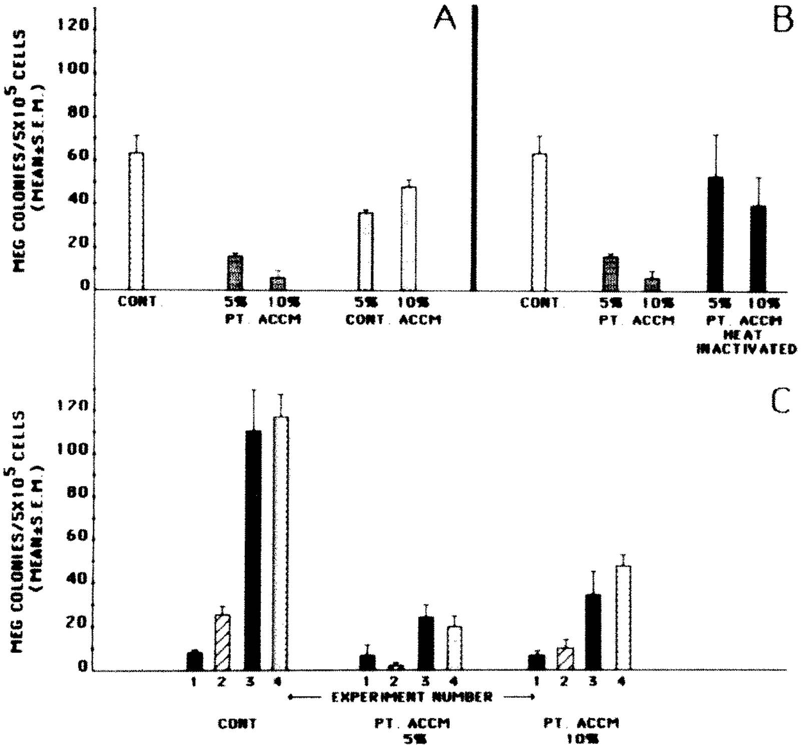

Fig 2. Effect of adherent cell conditioned medium (ACCM) of patient 2 on megakaryocyte colony formation by autologous and allogenic normal bone marrow mononuclear cells. Serum used to supplement the cultures was derived solely from normal AB donors. Results are expressed as mean ± SEM of colonies enumerated in duplicate or quadruplicate cultures. Panel A demonstrates the effect of the patient's ACCM on autologous megakaryocyte colony-forming unit (CFU-Meg) cloning efficiency. Highly significant inhibition was seen at the 5% (vol/vol) ($P < .02$) and 10% ($P < .01$) ACCM concentrations. No significant effect ($P > .05$) on megakaryocyte colony formation was observed in the presence of control ACCM at either the 5% or 10% concentration when assayed simultaneously against the same cells. Panel B demonstrates that CFU-Meg inhibitory activity was lost after the ACCM was heated for 30 minutes at 60 °C. The effect of the ACCM of patient 2 on four different normal donor-derived marrow mononuclear cells is shown in panel C. The number of colonies enumerated in the presence of ACCM, at both the 5% and 10% final concentration (vol/vol), differ significantly ($P < .05$) from control in experiments 2 through 4.

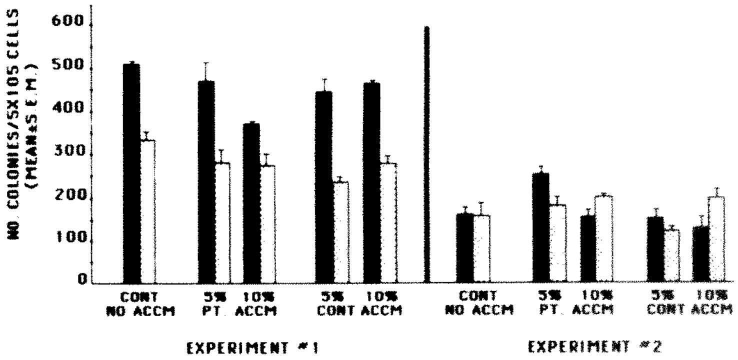

Fig 3. Effect of autologous adherent cell conditioned medium (ACCM) and normal control ACCM on CFU-E-derived (■) and BFU-E-derived (▨) colony formation by the marrow mononuclear cells of patient 2. Experiments 1 and 2 were performed on two different occasions. Cultures contained 2 IU/mL of erythropoietin (Connaught, Step III). Bars represent mean ± SEM of colonies enumerated in duplicate (CFU-E) or quadruplicate (BFU-E) culture platelets. Neither patient nor control ACCM had a significant effect on BFU-E-derived colony formation. Patient ACCM decreased autologous CFU-E colony formation by 29% at a final concentration of 10% (vol/vol) in experiment 1. The significance of this finding is unclear.

tions of 5% and 10% (vol/vol) were tested. In contrast to the effect of ACCM on megakaryocyte colony formation, patient ACCM did not significantly suppress ($P > .05$) BFU-E colony formation at the 5% or 10% concentration. At the 10% concentration, patient ACCM did result in a statistically significant ($P < .001$) 29% reduction in CFU-E–derived colony formation in comparison to control growth in the first of two experiments (Fig 3A); however, the same concentration of control ACCM also resulted in a significant ($P < .02$) albeit smaller 9% reduction in CFU-E–derived colonies. In no case did the observed decrement in erythroid colony formation approach that observed for megakaryocytes (Fig 2). The experiments depicted in Fig 2, panel A, and Fig 3, panel B, were conducted simultaneously using the same lot of patient ACCM and autologous target cells.

The effect of T lymphocyte depletion on megakaryocyte colony formation by marrow cells obtained from four normal controls and each of the patients is shown in Fig 1B. In aggregate, the data derived from studying the four normal individuals disclosed no significant effect on megakaryocyte colony formation as a result of this procedure. This was not true for the patients as, in each one, significant changes in cloning efficiency were noted after their marrow cells were T lymphocyte depleted.

In patient 1, T lymphocyte depletion led to the formation of 9 ± 0 (mean ± SEM of duplicate cultures) megakaryocyte colonies whereas essentially none were formed by unseparated mononuclear cells ($P < .001$). Furthermore, the unfractionated bone marrow cells of patient 1 did not increase colony formation in the presence of aplastic anemia serum (data not shown). Addition of the same amount of this same source of Meg-CSF to T depleted marrow mononuclear cells caused colony formation to double from 9 ± 0 to 18.5 ± 1.5 ($P < .001$). Because the number of T lymphocytes in this patient's unseparated mononuclear cell preparation was ~35%, removal of this cell type would have been expected to lead to the formation of 1.5 colonies/5 × 10^5 cells cloned. It is therefore unlikely that this observed increase in cloning efficiency was an artifact of the depletion procedure.

Removal of T cells from the marrow mononuclear cells of patient 2 totally abrogated megakaryocyte colony formation (Fig 1B). This effect could not be overcome by the addition of aplastic anemia serum to the cultures. It was not due to loss of CFUs, since culturing the T lymphocyte-rich cell fraction at 5 × 10^5 cells/mL never resulted in the formation of more than two megakaryocyte colonies.

Thus, the T cell depletion studies suggested that these cells might exert either a net suppressive (patient 1) or trophic (patient 2) influence on in vitro megakaryocytopoiesis. This hypothesis was tested by co-culturing recovered T lymphocytes with autologous T lymphocyte-depleted marrow mononuclear cells (Table 3). At a target cell/T cell ratio of 20:1, megakaryocyte colony formation by the marrow cells of patient 1 was decreased, but statistically unchanged, from colony formation observed in her T-depleted control cells. At ratios of 10:1 and 5:1, however, colony formation was suppressed 94% and 100%, respectively ($P < .01$). The opposite effect was noted on both occasions that patient 2 was studied. At target cell/T cell ratios of ≥10:1, a significant augmentation in megakaryocyte cloning efficiency was observed (Table 3). In contrast to these results, addition of

Table 3. Co-Culture Experiments With Autologous T Lymphocytes

T Lymphocyte/ Target Cell* Ratio	No. of Colonies Formed	Effect on Colony Formation
Patient 1		
T Cells alone	0.0 ± 0.0†	—
Target cells alone	9.0 ± 0.0	—
1:20	2.0 ± 1.4	↔ ($P > .05$)
1:10	0.5 ± 0.5	↓ 94% ($P < .01$)
1:5	0.0 ± 0.0	↓ 100% ($P < .01$)
1:2	—	NT
Patient 2		
T Cells alone	1.5 ± 0.7‡	—
Target cells alone	0.0 ± 0.0	—
1:10	8.3 ± 1.5	↑ 8-fold ($P < .001$)
1:5	—	NT
1:2	9.0 ± 1.1	↑ 9-fold ($P < .001$)
1:1	6.8 ± 1.6	↑ 7-fold ($P < .02$)
Controls§		
T Cells alone	0.5 ± 0.3	—
Target cells alone	39 ± 12	—
1:10	27 ± 8	↔ ($P > .05$)
1:5	19 ± 4	↔ ($P > .05$)
1:2	26 ± 7	↔ ($P > .05$)
1:1	35 ± 13	↔ ($P > .05$)

NT, Not tested.

*T Lymphocyte-depleted marrow mononuclear cells.

†Mean ± SEM of duplicate cultures.

‡Mean ± SEM of quadruplicate cultures.

§Aggregate results from four normal donors.

autologous T cells to four normal control marrows effected no significant ($P > 0.05$) change in colony formation.

Finally, we repeated the above studies on patient 1 when she was in the midst of a spontaneous recovery, and her platelet count was 92,000/μL. At the time, cloning efficiency of her unseparated marrow cells was still poor but, in contrast to the first study, T lymphocyte depletion had no discernible effect on megakaryocyte colony formation [3.8 ± 2.2 v 2.5 ± 2 colonies, respectively, per 5×10^5 mononuclear cells cloned ($P > .05$)].

DISCUSSION

Various bone marrow failure states, including aplastic anemia,[17-20] acquired pure RBC aplasia,[22,24] and selective neutropenia[25,26] have been associated with either T lymphocyte-mediated[17-19,24-27] or monocyte-macrophage-mediated[20,21] suppression of in vitro hematopoiesis. Our data implicating marrow accessory cells of both types in the suppression of in vitro megakaryocytopoiesis in some patients with AATP appears to be the first of its kind.

T lymphocytes of undetermined subtype appeared to be the suppressor-effector cells in the bone marrow of patient 1. This conclusion is based on a number of observations. First, T lymphocyte depletion augmented megakaryocyte colony formation by the patient's marrow mononuclear cells (Fig 1B). It is unlikely that this effect was due to concentration of megakaryocyte progenitor cells by the T cell depletion procedure, since the number of colonies formed was still significantly more than would have been expected based on the percentage of T lymphocytes present in the patient's unseparated marrow mononuclear cells. It is possible, however, that the observed increase in CFU-Meg cloning efficiency was due to the effect of concentration an unknown marrow accessory cell. Such a possibility is difficult to exclude but is also unlikely, since no increase in colony formation was noted after performing the same procedure in patient 2, who had a similar illness or in the normal controls (Fig 1B). In addition, autologous co-culture studies with the recovered T lymphocytes and T cell-depleted target cells resulted in suppression of colony formation at physiologic ratios (Table 3). Finally, during the recovery phase of the patient's illness, this in vitro phenomenon could no longer be demonstrated.

Unfortunately, we were unable to obtain sufficient marrow cells from patient 1 to demonstrate that the T cells involved suppressed only cells of the megakaryocyte lineage. Nevertheless, we still believe it is reasonable to assume that the suppression observed was specific since: (a) T cell-mediated suppression of erythropoiesis and granulopoiesis is well documented[22-26]; and (b) the patient's erythroid and granulocyte compartments, in distinct contrast to her megakaryocyte compartment, were intact. Had suppression not been specific, we would have expected to see aplasia of another line as well.

It is unclear why megakaryocyte colony formation by the recovering marrow cells of patient 1 remained so low, but persistent subnormal progenitor cell cloning efficiency in spite of clinical hematologic recovery has been reported in patients with aplastic anemia[21] and pure RBC aplasia.[22] Possible explanations include an absolute decrease in the number of marrow megakaryocyte progenitor cells or a more subtle form of damage than these assays can detect.

Quite different results were obtained in the second patient. In this case, suppression of megakaryocytopoiesis appeared to be due to the effect of adherent cells of the monocyte-macrophage lineage. This conclusion is based on reasoning similar to that applied to the case of patient 1. Megakaryocyte colony formation was augmented by depletion of adherent marrow cells (Fig 1A) and could not be attributed to nonspecific concentration of megakaryocyte progenitor cells by the depletion procedure. Again, the possibility that the observed increase in megakaryocyte colony formation was due to the effect of concentrating an unidentified marrow accessory cell must be considered but would appear unlikely in this case as well since colony formation was inhibited by co-culturing recovered adherent cells with the marrow mononuclear cells from which they had been removed (Table 2).

The suppressive effect of the MØ cells was noted at target/effector cell ratios similar to those reported by others to cause inhibition of erythropoiesis.[28-30] In addition, studies performed with ACCM suggested that the inhibitory factor was elaborated by these cells into their environment. Initial characterization of the inhibitor showed that it was heat labile, suggesting that it was not macrophage derived PGE_2,[31] or PGE_1, which is known to be even more heat stabile. We demonstrated that its activity was not MHC restricted since it inhibited allogenic as well as autologous CFU-Meg (Fig 2, panels A and C). Finally, the effect of this inhibitor appeared to be specific for cells of the megakaryocyte lineage, since no inhibitory activity against autologous BFU-E could be demonstrated on either of two separate occasions (Fig 3). The significance of the 29% inhibition of autologous CFU-E growth by the ACCM of patient 2 at the 10% (vol/vol) concentration in the first experiment (Fig 3, panel A) is unclear. Some degree of inhibition was also noted with control ACCM in this experiment as well, and in neither case did the suppression observed approach the 50% Δ value that some experts require before imparting biological significance to such an observed effect.[32]

In cases of bone marrow failure attributed to humoral inhibitors, progenitor cell cloning efficiency in vitro may be less perturbed[32-34] than in those in which cellular mechanisms are implicated.[19,22] With this consideration in mind, the number of residual megakaryocyte progenitors detected in the bone marrow of patient 2 was somewhat surprising. Bagby and co-workers have demonstrated that these associations are not absolute, however, by showing significant granulocyte-macrophage colony formation in some patients with apparent T cell-mediated granulopoietic failure.[25,26]

In addition, humoral suppression of hematopoiesis is often demonstrable by co-culturing patient marrow in fresh, autologous plasma or serum that has not been heat inactivated.[2,33,34] Such a technique was used in the first description of antibody-mediated AATP.[2] In that patient, addition of fresh autologous serum to her bone marrow cultures suppressed megakaryocyte colony formation. Screening of the serum of patient 2 for potential inhibitors, using the same technique, resulted in marked stimulation of her own (Table

1) as well as other normal donors' megakaryocyte progenitor cells (data not shown). Accordingly, we feel that the existence of a co-existing humoral inhibitor in this patient was unlikely. In patient 1, this question could not be reliably addressed since her cloning efficiency was so poor.

Our results also suggest that the role of T lymphocytes in the regulation of human megakaryocytopoiesis in vitro is complex. These cells may exert either suppressive or trophic effects on megakaryocyte progenitor cells (Fig 1, Table 3). Thus, the CFU-Meg of patient 1 appeared to be inhibited by T cells, whereas in patient 2, CFU-Meg-derived colony formation fell to zero when T cells were selectively removed from her marrow mononuclear cells (Fig 1B). This result could not be attributed to procedure-induced loss of megakaryocyte progenitors and was presumably caused instead by unopposed suppressor activity of the patient's adherent marrow cells. By implication, T lymphocyte–monocyte interactions may have been important in determining net megakaryocyte colony formation in vitro and perhaps the growth of megakaryocyte progenitor cells in vivo as well. We also noted that when the T lymphocytes of patient 2 were co-cultured with autologous marrow mononuclear cells, marked stimulation of megakaryocyte colony formation occurred (Table 3). These data also support the hypothesis that T cells may exert a trophic influence on progenitor cells of the megakaryocyte compartment and may cooperate with other ancillary bone marrow cells in regulating their growth. The recent report by Geissler and co-workers[35] that phytohemagglutin–leukocyte conditioned medium (PHA-LCM)–activated T cells stimulate CFU-Meg proliferation in an agar culture system would appear to support this hypothesis.

Recent reviews[36,37] have pointed out that the body of evidence implicating immune effector cells in the regulation of erythropoiesis and granulopoiesis is both large and compelling. Little is presently known regarding the cellular regulation of human megakaryocytopoiesis under pathologic or normal conditions. Further studies on the cellular interactions regulating growth and development of CFU-Meg are therefore likely to reveal important insights into the pathogenesis of as yet poorly characterized disorders of megakaryocyte and platelet production.

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Quantitative Analysis of Platelet Surface HLA by W6/32 Anti-HLA Monoclonal Antibody

By K.J. Kao, Daniel J. Cook, and Juan C. Scornik

Class I molecules of human major histocompatibility complex (HLA) are the most important antigenic system in determining the survival of transfused platelets in alloimmunized patients. Platelets with reduced expression of a specific type of HLA antigen may escape specific anti-HLA antibody-mediated destruction. By using ^{125}I-labeled Fab fragments of W6/32 anti-HLA monoclonal antibody and competitive protein binding assays, we measured the range of total HLA concentrations on platelets. In 12 individuals examined, the mean number of HLA-A, B, and C molecules per platelet was 81,587 ± 20,016 (mean ± SD); its range was between 54,782 to 116,185 molecules per platelet. After treatment with chloroquine, 79.9 ± 7.0% (mean ± SD, n = 6) of HLA antigens were removed from platelets as determined by binding of ^{125}I-W6/32 Fab. A similar result was obtained when HLA antigens on chloroquine-treated platelets were evaluated with immunofluorescence flow cytometry. In contrast, chloroquine treatment did not remove integral membrane protein such as P1^{A1} antigens on platelets. The presence of HLA antigens in the chloroquine eluate of platelets could be demonstrated to contain HLA antigens similar in mol wts to intact class I molecules by an immunoblotting technique. These data suggest that 70% to 80% of platelet HLA antigens are adsorbed and that such HLA antigens are not proteolytic products of integral membrane class I molecules. The origin of the adsorbed platelet HLA-antigens remains to be determined.

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THE EXISTENCE OF HLA antigens on platelets was first demonstrated with platelet complement-fixation tests in the 1960s[1-5] and was later confirmed with the purification of HLA antigens from platelets.[6,7] Clinically, HLA is the most important antigen system involved in determining survival of transfused platelets in alloimmunized patients.[8-13]

Liebert and Aster and co-workers[14-18] used an inhibition of lymphocytotoxicity test to show that several B-locus HLA antigens of platelets are variably expressed among different individuals. A threefold variation of HLA-A2 on platelets was also reported by Svejgaard.[19] HLA-C antigens are only weakly expressed on human platelets[20] and do not seem to play any significant role in the alloimmunization to platelets.[21] In addition, platelets with reduced expression of HLA-B12 may escape destruction and survive normally in recipients whose plasma contains antibodies against HLA-B12.[9,16,22,23] These observations suggest that quantitative difference of HLA antigen expression may have a potential clinical importance and stress the need for more rigorous studies to improve methods of quantitation over serological techniques relied upon by previous investigations.[14-19]

Recently, Blumberg and colleagues[24] reported that acid hypertonic chloroquine can elute a portion of HLA antigens without affecting P1^{A1} antigens on the platelet surface. The findings indicated that certain amounts of HLA antigens on the platelet surface were adsorbed. However, the exact quantity was not determined.

In this report, we describe methods of quantitation of platelet surface HLA antigens and show the elutability of these antigens and variations of HLA concentrations on platelets of normal individuals.

MATERIALS AND METHODS

Materials. RPMI 1640 medium, L-cysteine, antibiotic-antimycotic solution, and fetal calf serum (FCS) were purchased from GIBCO Laboratory (Grand Island, NY). Protein A-Sepharose CL 4B, mercuripapain, bovine serum albumin (BSA), nitroblue tetrazolium (NBT), 5-Bromo 4-chloro 3-indolyl phosphate *p*-toluidin salt (BCIP), rabbit anti-mouse IgG alkaline phosphatase conjugate, and chloroquine diphosphate were obtained from Sigma (St Louis). Na^{125}I and solid-phase lactoperoxidase were the products of Amersham Co (Arlington Heights, Ill) and P.-L. Biochemicals (Milwaukee), respectively. Fluorescein-labeled sheep anti-mouse IgG F(ab') 2 fragments and mouse IgG were obtained from Cooperbiomedical, Inc (Malvern, Pa). Goat anti-human IgG F(ab') 2 fragments were from Kallestad Laboratory (Austin, Tex). Diethylaminoethyl (DEAE) affi-gel blue and nitrocellulose paper with 0.45-μm pore size were purchased from Biorad Co (Richmond, Calif). All other chemical reagents were analytical grade. Mouse anti-influenza virus type A monoclonal antibody was a generous gift from Dr P.A. Klein, Department of Pathology, University of Florida.

Isolation of W6/32 anti-HLA monoclonal antibody. The W6/32 hybridoma, obtained from the American Type Culture Collection (Rockville, Md), was originally developed and had been shown to recognize a common monomorphic determinant of HLA-A, B, and C molecules by Barnstable and colleagues.[25] The W6/32 hybridoma was maintained in RPMI 1640 culture medium supplemented with 10% FCS, gentamycin (0.5 mg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL). W6/32 anti-HLA monoclonal antibody was purified from culture medium by protein A-Sepharose CL-4B affinity column chromatography.[26] Protein concentration and homogeneity of the purified W6/32 monoclonal IgG antibody were determined by the assay of Lowry and co-workers[27] and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[28] respectively.

Preparation of Fab fragments of W6/32 monoclonal antibody. The Fab fragments of W6/32 anti-HLA monoclonal antibody (W6/32-Fab) were prepared by papain digestion as described by Porter.[29] In brief, 20 mg of W6/32 antibody in 5 mL of 0.01 mol/L of potassium phosphate buffer, pH 6.5, containing 2 mmol/L of EDTA and 0.01 mol/L of L-cysteine were incubated with 0.4 mg mercuripapain at 37 °C for 4 hours. The papain digest of W6/32 antibody was dialyzed with 0.02 mol/L of Tris-HCl, pH 8.0, containing 0.025 mol/L of NaCl at 4 °C for 10 hours and then

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loaded on a DEAE affi-gel blue column (1.5 × 10 cm) to separate Fab fragments from Fc fragments. The Fab fragments were recovered from the protein peak in the flowthrough and further purified with a protein A-Sepharose CL 4B column chromatography (1.5 × 3 cm) described above.[26]

Preparation of washed platelets. Only freshly drawn venous blood from healthy volunteer donors was used. Blood was anticoagulated with 1/9 vol of 3.8% sodium citrate. Platelets were isolated from 18 mL of the blood by differential centrifugation.[30] The isolated platelets were washed twice with 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.15 mol/L of NaCl and 1 mmol/L of EDTA (Tris-saline-EDTA) as described,[31] and finally suspended in 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.15 mol/L of NaCl and 0.2% bovine serum albumin (BSA) (Tris-saline BSA). The platelet concentrations were enumerated by an S-plus Coulter counter. The contamination of WBCs was always <0.03%.

Chloroquine elution of platelet HLA. The chloroquine solution was prepared as reported by Blumberg and co-workers.[24] In brief, 20g of chloroquine diphosphate was dissolved in 100 mL of 0.01 mol/L of sodium phosphate buffer, pH 7.4, containing 0.1 mol/L of NaCl. The pH was then adjusted to 5.0 ± 0.1 by 5 N NaOH. Four volumes of chloroquine diphosphate were mixed with 1 vol of washed platelets (3×10^9 cells/mL) suspended in Tris-saline without BSA and incubated at 25 °C for 60 minutes. After incubation, chloroquine-treated platelets were collected by centrifugation at 2,200 g for 10 minutes, washed twice with Tris-saline EDTA, and suspended in Tris-saline-BSA for later assay.

Quantitative assay for platelet HLA-A, B, and C antigens. The concentration of total HLA-A, B, and C antigens on platelets was determined by using ^{125}I-labeled Fab fragments of W6/32 anti-HLA monoclonal antibody (^{125}I-W6/32 Fab) and a competitive protein binding assay.[32,33] The isolated W6/32 Fab was labeled with ^{125}I by solid-phase lactoperoxidase method as described previously.[34] The specific radioactivity of ^{125}I-W6/32 Fab was ~0.6 μCi/μg. The specific bindings of ^{125}I-W6/32 Fab to washed platelets were determined by incubating 5×10^6 platelets and specified amounts of ^{125}I-W6/32 Fab in 250 μL of Tris-saline-BSA with or without a 160-fold excess of unlabeled W6/32 Fab. After 2 hours of incubation at room temperature, the incubations were terminated by adding 0.5 mL of ice-cold Tris-saline-BSA, and they were centrifuged at 2,200 g for 10 minutes at 4 °C. The addition of 0.5 mL of ice-cold Tris-saline-BSA reduced the nonspecific binding without any significant effect on the specific binding. The supernatant of each incubation was aspirated, and the platelet pellet was counted in an LKB gamma counter. The ^{125}I-W6/32 bound on platelets which could be competed only by an excess of unlabeled W6/32 Fab was defined as specific binding. All experiments were performed in duplicate. The variation between duplicates was <7%. The specific binding of ^{125}I-W6/32 to platelets was not affected by the presence or absence of 0.05% sodium azide in the incubation mixtures.

Immunofluorescent flow cytometry. Immunofluorescent flow cytometry was conducted on a Becton-Dickinson FACS-II fluorescence-activated cell sorter. Routinely, 1×10^7 washed platelets were incubated with saturable amounts of primary antibody, either 10 μg/mL of W6/32 monoclonal antibody or 20 μL of anti-Pl^{A1}antiserum in a final volume of 100 μL of Tris-saline at room temperature for 30 minutes. Thereafter, the platelets were washed three times with 0.5 mL of Tris-saline-EDTA and reincubated with 50 μL of fluorescein-labeled F(ab')2 of anti-human or mouse IgG at 1 to 20 dilution for 20 minutes at room temperature. Finally, the platelets were washed twice and resuspended in 1 mL of 0.01 mol/L of sodium phosphate buffer, pH 7.4, containing 0.15 mol/L of NaCl (phosphate buffer saline, PBS). The fluorescent intensity of 10,000 platelets was measured. The histogram was depicted, and the mean fluorescence intensity per platelet was computed with a Hewlett-Packard 9845T computer.

SDS-PAGE and Western blot. SDS-PAGE was performed according to the procedure of Laemmli.[28] The supernatant of washed platelet suspension (1×10^9 cells/mL) that was treated with 4 vol of chloroquine was collected by centrifugation at 4,000 g for 10 minutes, and dialyzed extensively with Tris-saline. The supernatant was then concentrated 75-fold with Aquacide II and Amicon B15 concentrator and was mixed with an equal volume of sample buffer for SDS-PAGE. Control washed platelets and chloroquine-treated washed platelets were solubilized by incubating 1×10^9 platelets with 0.3 mL of 0.5% Nonidet-P40 in PBS and containing 1 mmol/L phenylmethylsulfonyl-fluoride and 1% epsilon-amino-*n*-caproic acid for 15 minutes on ice. Insoluble cellular debris was removed by centrifugation at 10,000 g for 5 minutes. The solubilized platelets were concentrated threefold with an Amicon B15 concentrator and mixed with an equal volume of sample buffer for SDS-PAGE.

Western blot was performed by following the procedure of Tobin and co-workers[35] in a Trans-Blot cell (Bio-Rad, Richmond, Calif). The blotting process was conducted at constant voltage of 40 V for 4.5 hours. The nitrocellulose blot was blocked with 5% nonfat milk made in PBS containing 0.05% sodium azide[36] and incubated sequentially with W6/32 anti-HLA antibody (20 μg/mL), alkaline phosphatase conjugate of rabbit anti-mouse IgG (1:1,000 dilution), and alkaline phosphatase substrate, which contains 0.1 mg/mL of NBT and 0.05 mg/mL of BCIP as described.[37]

RESULTS

SDS-PAGE of W6/32 monoclonal antibody and W6/32 Fab. The mouse W6/32 anti-HLA monoclonal antibody is an IgG antibody and reacts specifically with a common determinant of HLA-A, B, and C molecules.[25] As shown in Fig 1, the isolated W6/32 anti-HLA monoclonal antibody appears to be homogeneous and contains equal amounts of two light chains as described previously.[25] One of the light chains is the parental myeloma type. The purified Fab fragments of W6/32 monoclonal antibody are homogeneous, as shown in Fig 1, and have a mol wt of 46 kd. After reduction with mercaptoethanol, Fab fragments also contain

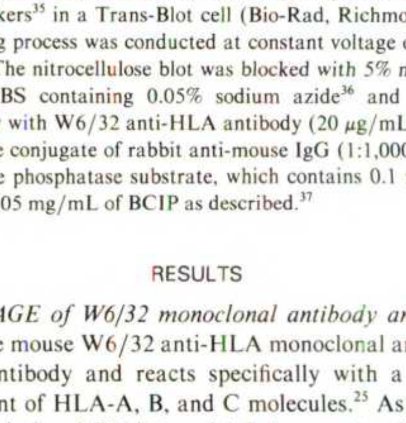

Fig 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of purified W6/32 anti-HLA monoclonal antibody and its Fab fragments. The polyacrylamide gel was 10%. Lane A, reduced W6/32 antibody (12 μg); lane B, reduced W6/32 Fab (10 μg); lane C, nonreduced W6/32 antibody (10 μg); lane D, nonreduced W6/32 Fab (10 μg). Left lane, mol wt standards: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

equal amounts of two intact light chains. The findings suggest that only 50% of W6/32 Fab may be functional and will react with HLA-A, B, and C molecules. This was experimentally tested by incubating ^{125}I-W6/32 Fab (1 µg/mL) and platelets (2.0 × 10^9 cells/mL) that contained an excess of HLA antigens in the presence or the absence of 150 µg/mL of unlabeled W6/32 Fab at 25 °C for 3 hours. All the incubations were performed in duplicate. The results showed that 47% of ^{125}I-W6/32 Fab specifically bound to platelets, thereby confirming that ~50% of the Fab fragments have HLA binding activity.

Quantitative Assay for Platelet HLA-A, B, and C Antigens. The purified W6/32 Fab was labeled with ^{125}I and used to quantitate platelet HLA concentrations in a competitive protein binding assay.[32,33] Fab fragments were used to avoid binding to platelet Fc receptors. First, the specific binding of ^{125}I-W6/32 Fab to 5 × 10^6 platelets was studied as a function of duration of incubation. The results showed that 2 hours of incubation is required for the binding to reach a steady state (Fig 2). Thereafter, specific binding was determined as a function of increasing concentrations of ^{125}I-W6/32 Fab after a 2-hour incubation time. As shown in Fig 3, the specific binding of ^{125}I-W6/32 Fab to a constant number of platelets was saturable. As expected, the nonspecific binding increased linearly with increasing concentrations of ^{125}I-W6/32 Fab and was not saturable.[33] The binding data of Fig 3 were then analyzed by a Scatchard plot[38] to determine the steady-state dissociation constant (Kd) and the concentration of total platelet HLA in the incubations. The results showed that the Kd was 6.1 nmol/L and total platelet HLA concentration was 3.35 nmol/L or 100,835 molecules per platelet (Fig 4).

To demonstrate the specificity of ligand binding, human fibrinogen and IgG from human and mouse were used to compete with ^{125}I-W6/32 Fab for binding. As shown in Fig 5, only unlabeled W6/32 Fab can compete for the binding of labeled W6/32 Fab. The results in Fig 5 were also analyzed by Scatchard plot. Similar binding affinity (Kd) and concentration of platelet HLA molecules to those determined from the binding data of Fig 3 were obtained. In these studies (Figs 3 and 5), washed platelets from the same individual were used. The results indicate that both methods are precise quantitations of platelet HLA concentration.

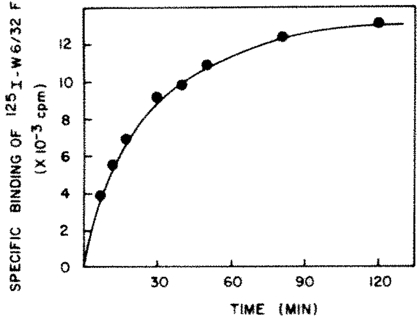

Fig 2. Time course study of binding of ^{125}I-labeled W6/32 Fab to platelets. Each incubation contained 5 × 10^6 platelets and 0.075 µg ^{125}I-W6/32 Fab (1.08 × 10^6 cpm/µg) with or without a 160-fold excess of unlabeled W6/32 Fab in a final volume of 250 µL. The bound ^{125}I-W6/32 Fab that could not be unlabeled W6/32 Fab represented nonspecific binding.

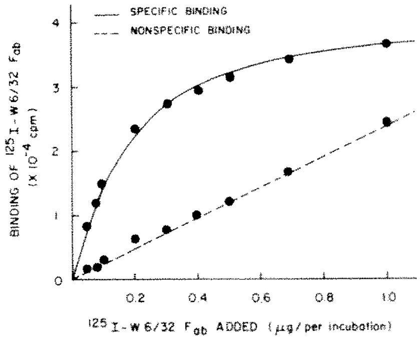

Fig 3. Saturable binding of ^{125}I-W6/32 Fab to platelets. The specific binding and the nonspecific binding of ^{125}I-W6/32 Fab to a constant number of platelets (5 × 10^6 cells) were determined as a function of increasing concentrations on ^{125}I-W6/32 Fab.

The specific bindings of ^{125}I-W6/32 Fab to human lymphoblastoid cells, platelets, Daudi cells (a Burkitt's lymphoma cell line), and RBCs were also determined to demonstrate that the binding of ^{125}I-W6/32 Fab is specific for class I HLA molecules. Specific binding per 5 million cells was observed in a lymphoblastoid cell line (28,035 cpm) and platelets (11,836 cpm) but not in RBCs (500 cpm) and Daudi cells (191 cpm). The results agree with earlier studies demonstrating that lymphoblastoid cells are rich in HLA antigens,[39] while HLA antigens are low or absent in Daudi cells[40] and RBCs.[41]

The interassay variation for quantitating platelet HLA concentration by using ^{125}I-W6/32 Fab and Scatchard analysis was also evaluated.[42] HLA concentrations on platelets

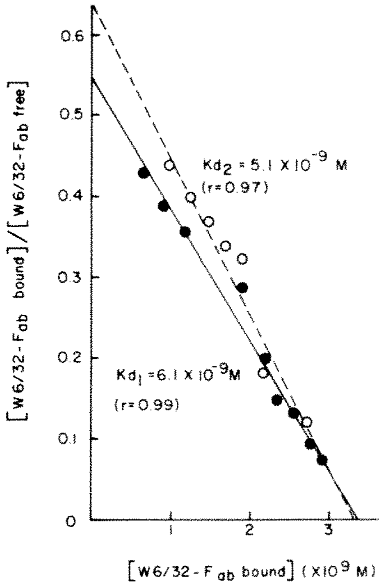

Fig 4. Scatchard plots of ^{125}I-W6/32 Fab binding to platelets. The binding data obtained from the studies of Fig 3 (—●—) and Fig 5 (---○---) were analyzed. The lines were drawn by linear regression analysis. The assumption that only 50% of added W6/32 Fab are functional was used for Scatchard analyses. The mol wt of 46 kd for W6/32 Fab was used for calculation. Kd, steady-state dissociation constant; *r*, correlation coefficient.

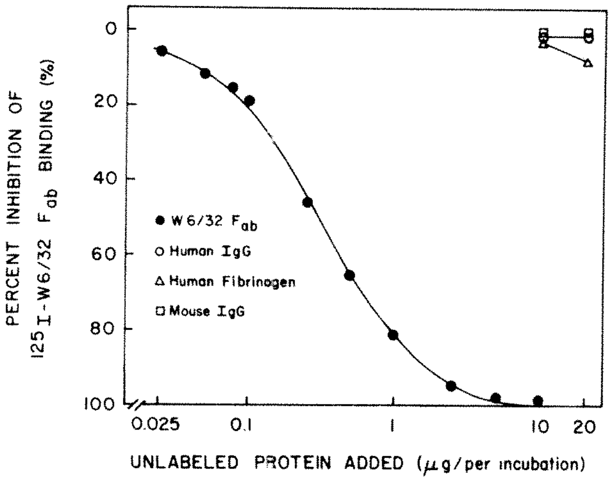

Fig 5. Competition study of ^{125}I-W6/32 Fab binding to platelets. The experiment was performed by incubating 5 × 10^8 platelets and 0.075 μg of ^{125}I-W6/32 Fab with specified concentrations of unlabeled proteins. The total specific binding was the difference of bound ^{125}I-W6/32 Fab between incubations with and without 20 μg of unlabeled W6/32 Fab. The total specific binding was defined as 0% inhibition.

harvested from three different persons were determined individually after their immediate collection and on the second and the third day were stored in Tris-saline containing 0.05% sodium azide at 4 °C. The interassay variations for each individual were 6.3%, 6.7%, and 6.8%, respectively.

Platelet HLA concentrations in normal adults. Platelet HLA concentrations in 12 healthy adults were determined by the established binding assay. All except 1 of the 12 adults were HLA typed. Amont these 12 individuals, the average number of HLA-A, B, and C molecules per platelet varied between 54,782 to 113,068 molecules per platelet (Table 1). The mean number of HLA molecules on a platelet was 81,587 ± 20,016 (mean ± SD). The results indicate that there is at least a twofold variation in platelet HLA concentration among different individuals. Because the interassay variation is 7%, the observed twofold difference most likely reflects true individual variation.

Quantitation of HLA molecules on chloroquine-treated platelets. The specific bindings of ^{125}I-W6/32 Fab to platelets pretreated with 4 vol of 20% chloroquine for 15 to 60 minutes were determined. It was noted that loss of HLA antigens reached its maximum after 45 minutes of incubation with chloroquine. The elution of platelet HLA antigens was then studied as a function of chloroquine concentration, and all platelets were incubated with chloroquine for 45 minutes. The results of these studies indicated that maximal elution was attained after incubating platelets with 4 vol of 20% chloroquine for 45 minutes. Subsequently, we performed experiments in six healthy individuals whose platelets were pretreated with 4 vol of 20% chloroquine for 60 minutes. Losses of 71.5% to 90.0% of HLA-A, B, and C molecules from the platelets were observed (Table 2). Although chloroquine treatment reduced HLA antigens, Pl^{A1} antigens remained unchanged on platelets, as determined by the binding of anti-Pl^{A1} antiserum and quantitative immunofluorescence flow cytometry. The mean fluorescence intensity for HLA antigen on each of the chloroquine-treated platelets was only 25.3 ± 9.3% (mean ± SD) of the control.

Table 1. Concentrations of Platelet HLA in Normal Adults

Case No.	Sex	HLA Phenotype	HLA-A, B, C (Molecules/per Platelet)*
1	M	2,24,62,39	100,835
2	M	24,—,7,—	66,220
3	M	26,28,14,53	60,200
4	M	1,3,7,8	90,300
5	M	1,2,7,12	65,317
6	M	2,28,17,40	116,185
7	M	24,30,7,14	79,765
8	F	ND†	54,782
9	F	2,23,8,44	72,240
10	F	24,30,18,44	79,463
11	F	2,32,4,37	113,068
12	F	3,24,7,8	80,667
			81,587 ± 20,016 Mean ± SD

*The concentrations of HLA-A, B, and C molecules were determined from Scatchard analyses of the binding studies.

†HLA phenotype was not done on case no. 8.

Western blot of platelet HLA and chloroquine eluate. To demonstrate whether the eluate of chloroquine contains HLA antigens lost from platelet surface, Western blot was used. The HLA antigens transblotted to nitrocellulose paper was detected by W6/32 monoclonal antibodies and alkaline-phosphatase conjugate of anti-mouse IgG antibodies. The results in Fig 6 show the presence of HLA antigens in the eluate of chloroquine-treated platelets with the same apparent mol wt of 45 kd as the intact membrane Class I HLA molecules. When the similar blot was incubated with control mouse anti-influenza virus type A monoclonal antibody, HLA antigens were not detected.

DISCUSSION

Although the presence of HLA antigens[1-5] and variable expression of some specific types of HLA antigens on human platelets[14-19] are known, the exact quantity of HLA molecules on platelets and its variability among different individuals has not been demonstrated. By using Fab fragments of W6/32 anti-HLA monoclonal antibody and competitive protein binding, a quantitative assay for determining concentration of platelet HLA-A, B, and C molecules was devel-

Table 2. Effect of Chloroquine Treatment on Platelet HLA Concentration

Case No.	HLA Concentration (Molecules/per Platelet)*		HLA Eluted (%)
	Control	Chloroquine Treated	
1	72,240	16,555	77.1
2	54,782	9,933	81.9
3	65,317	19,264	71.5
4	79,765	12,130	84.8
5	113,068	29,623	73.8
6	80,667	8,066	90.0
Mean ± SD	77,639 ± 19,861	15,928 ± 7,889	79.9 ± 7.0

*The HLA-antigen concentration on platelets were determined by ^{125}I-W6/32 Fab binding studies and Scatchard analyses.

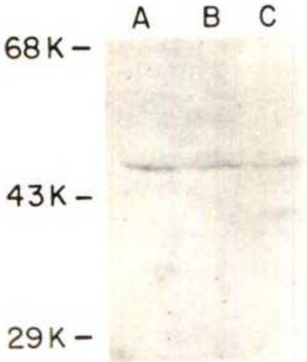

Fig 6. Western blot of HLA antigens from control platelets, chloroquine-treated platelets, and chloroquine eluate. The solubilized platelets and chloroquine eluate were prepared as described in the Materials and Methods section. Each sample that contained solubilized proteins or eluted proteins from $\sim 2 \times 10^8$ cells was analyzed on a 7.5% to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The HLA antigens were detected with W6/32 monoclonal antibody and alkaline phosphatase conjugate of rabbit anti-mouse IgG antibody after the proteins were transferred to nitrocellulose paper electrophoretically. Lane A, control platelets; lane B, eluate of chloroquine-treated platelets; lane C, chloroquine-treated platelets. The similar blot was incubated with mouse anti-influenza virus type A monoclonal antibody as a control. No binding of the antibody was detected.

oped. The saturability of binding, ligand and cellular specificity, and high binding affinity substantiate the validity of this assay.

As reported previously,[25] the W6/32 monoclonal antibody contains equal amounts of two different light chains and reacts with a common monomorphic antigenic determinant on HLA-A, B, and C molecules. One of the light chains is parental myeloma type.[25] Therefore, it is expected that only 50% of Fab fragments will react with HLA antigens, a fact that we confirmed experimentally. This was taken into consideration when the binding data were analyzed by Scatchard plot. The linear Scatchard plots indicated that only one class of binding sites on platelets and Fab fragments was apparently homogeneous in binding.[32,33]

The results of studying platelets from 12 healthy individuals revealed that at least a twofold variation exists between the lowest and highest platelet HLA concentrations. This finding is in parallel with the reported variability for platelet HLA-A2, B5, B7, B27, or B13[14,18,19] but not for B12 and B8.[14] A 35-fold and an eightfold variation was reported for platelet HLA-B12 and B8, respectively.[14] Most of these previous studies using inhibition of lymphocytotoxicity measured the relative rather than the exact quantity of platelet HLA antigen. Although the degree of inhibition can be proportional to the actual quantity of HLA antigens, other factors, such as presence of other HLA antibodies and cross-reactivity with other HLA antigens, may affect the results.

Although there was no apparent association between platelet HLA concentration and specific HLA phenotype, the number of individuals ($n = 12$) involved in the present study was small. Further study on a larger number of individuals will provide a more definite conclusion. The mean number of HLA-A, B, and C molecules on a platelet as determined in our studies is almost the same as that on a peripheral blood T lymphocyte[39] and about one-third of that on a peripheral blood B lymphocyte.[39] Considering the size of platelets (2 to 3 μm in diameter) and lymphocytes (6 to 14 μm in diameter), it appears that the density of HLA antigen on the platelet surface is greater than that on peripheral blood lymphocytes.

Recently, Blumberg and co-workers[24] reported a reduction of platelet surface HLA antigens after acid hypertonic chloroquine treatment of platelets. However, the exact quantity of HLA antigens reduced by chloroquine was not clear. By using ^{125}I-W6/32 Fab and competitive protein binding assay, we found that 70% to 80% of platelet HLA antigens could be removed by chloroquine (Table 2). Because the presence of HLA antigens was identified in the chloroquine eluate by immunoblotting (Fig 6) and because treatment of chloroquine did not significantly affect platelet $\mathrm{P1}^{\mathrm{A1}}$ antigens which are part of the integral membrane glycoprotein IIIa.[43] These results strongly suggest that most platelet HLA antigens are adsorptive in nature. Moreover, the elutable HLA antigens appear to have the same mol wts as do intact HLA molecules (Fig 6). Thus, it is unlikely that they are proteolytic products of intact membrane HLA molecules. Previously, Lalezari and Driscoll[44] demonstrated that platelets may acquire HLA antigens from plasma. The possible contribution of plasma HLA antigens to those adsorbed on platelets remains to be determined.

In conclusion, the results of our studies indicated that human platelets contain high concentrations of HLA antigens. More important, most HLA antigens are adsorbed on platelets. Thus, further studies on the origin and the binding mechanism of adsorbed platelet HLA antigens may lead to the development of ways to manipulate platelet HLA antigens quantitatively and qualitatively. By such manipulation, one may be able to reduce the platelet immunogenicity for their use in patients with alloimmunization against HLA antigens in the future.

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Inducible Production of Human Macrophage Growth Factor, CSF-1

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A panel of human cell lines was screened for production of colony-stimulating factor-1 (CSF-1) using a specific radioreceptor assay and criterion of macrophage colony growth in mouse bone marrow culture. The pancreatic carcinoma lines MIA PaCa and PANC were found to secrete high levels of CSF-1. In a bone marrow proliferation assay, the activities from these two lines were blocked by a CSF-1 specific neutralizing antiserum, confirming the predominant content of this macrophage growth factor. MIA PaCA cells stopped secreting CSF-1 when transferred to various serum-free media. Serum-free production could be reinitiated by phorbol myristic acetate (PMA). Purified CSF-1 from serum-free MIA PaCa cells stimulated the formation of 14-day colonies from total and nonadherent mononuclear human bone marrow cells. Most of the colonies consisted exclusively of large, dispersed macrophages that were intensely stained for nonspecific esterase. Although similar numbers of human 14-day colonies were stimulated by CSF-1 and other CSFs, more CSF-1 was required for the proliferation of human as compared with murine bone marrow progenitors. Northern analysis of mRNA from induced-MIA PaCa cells, using a human CSF-1 oligonucleotide probe, revealed multiple species of CSF-1 mRNA ranging from 1.5 to 4.5 kilobases (kb). Uninduced, serum-free cultures showed only the largest mRNA species, suggesting that serum removal interfered with CSF-1 mRNA processing related to synthesis and/or secretion of the protein. Regulation of the production of CSF-1 may be an important physiological process in hematopoiesis and macrophage functioning.

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THE GENERATION OF granulocytes and macrophages from immature hemopoietic progenitor cells is dependent on the presence of several hormone-like, glycoprotein growth factors, the colony-stimulating factors or CSFs.[1] Several subclasses of human CSFs can be distinguished by the kind of mature cells they produce in semisolid culture media. One subclass, CSF-1, stimulates predominantly monocyte/macrophage production.[2]

Native human urinary CSF-1 is a glycosylated homodimer with a mol wt of ~45,000.[3] It can be clearly distinguished from the other subclasses by specific radioimmunoassays and radioreceptor assays as well as by its target cell specificity for growth.[2] Specific cell surface receptors which mediate the biological effects of CSF-1 are selectively expressed on mononuclear phagocytic cells.[4] Wu and coworkers[5,6] described a pancreatic carcinoma cell line, MIA PaCa, and other sources of a CSF chemically similar to the human urinary CSF-1 that induced macrophage colonies in murine bone marrrow cultures.

We recently cloned the human CSF-1 gene and expressed the complementary DNA in primate cells.[7] In this report, we describe regulation of human CSF-1 release and CSF-1 mRNA expression in serum-free cultures of the MIA PaCa line, and aspects of the action of this CSF-1 on murine and human progenitor cells.

MATERIALS AND METHODS

Cell lines. Pancreatic carcinoma lines MIA PaCa-2[6] and PANC-1[8] were obtained from the American Type Culture Collection, Rockville, Md. The bladder carcinoma line 5637[9,10] and L929[2] were described previously. Supernatants of GCT[11] were purchased (GIBCO, Grand Island, NY).

Murine bone marrow assays. Bone marrow colony formation[2] was measured using mononuclear bone marrow cells from Balb/c mice. The cells were incubated for 7 days in MEM α-medium–10% fetal calf serum (FCS) containing 0.3% agar (10^5 cells in 1 mL plus 0.1 mL of CSF, in six well plates). Colonies (>50 cells) were counted, and some were removed from the agar individually, cytocentrifuged, and stained.

The mouse bone marrow thymidine-incorporation assay has been described previously.[12] In brief, mononuclear bone marrow cells from Balb/c mice were incubated for 3 days in dilutions of CSF in α-medium. The cell number was 5×10^4 cells per well in 0.2 mL in 96-well plates. The cells were pulsed with ^{3}H-thymidine for 6 hours (50 μCi per well), harvested on glass fiber filters and assayed for radioactivity by liquid scintillating counting. A partially purified preparation of CSF-1 from MIA PaCa supernatant, quantified by the mouse bone marrow colony assay, was used as a standard.

CSF-1 radioreceptor assay. The radioreceptor assay is a highly specific assay for CSF-1 from human and murine sources, and was run with slight modification from that previously described.[2,13] In brief, the mouse macrophage cell line J774.2, which expresses CSF-1 receptors, was incubated for 1 to 1½ hours at 4 °C with dilutions of CSF-1 samples in 96-well plates (1.3×10^5 cells per well). Iodinated purified mouse L cell CSF-1 was added and incubated for 1 hour at 4 °C. The cells were harvested on glass fiber filters with extensive washing, and the filters were counted. Partially purified preparations of human urinary CSF-1 and MIA PaCa CSF-1, quantified in the mouse bone marrow colony assay, were used as standards.

Human bone marrow colony assay. Low-density bone marrow cells from normal volunteers were prepared by differential centrifugation in bovine serum albumin, density 1.070 g/cm^3.[14] Adherent cells were removed by culture on plastic tissue dishes at 10^6 cells/mL for 1.5 hours. The CFU-GM assay in agar was described previously.[14] Conditioned medium from the 5637 cell line was used as a control source of CSFs.

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Partial purification of CSF-1 from MIA PaCa cells. Cells grown to confluence in roller bottles in 10% FCS were washed and reincubated in protein-free Dulbecco's Modified Eagle's Medium containing 10 ng/mL of phorbol myristic acetate (PMA) and 10 μmol/L of retinoic acid for 3 to 4 days. The supernatant was centrifuged and concentrated by adsorption to calcium phosphate gel,[3] and the eluted CSF-1 (fraction II) was further purified by affinity chromatography on a lentil-lectin Sepharose column (fraction III).[14a] Fractions II and III had specific activities of $\sim 10^4$ and 10^5 U/mg, respectively, by mouse bone marrow colony assay, and $>90\%$ of the bone marrow stimulatory activity was CSF-1, as shown by colony morphology and by inhibition by a specific neutralizing rabbit antiserum[2] (Tables 1 and 2). The LPS content was <0.1 ng/1,000 U of CSF-1, measured by the Limulus assay.[40]

mRNA isolation and Northern gel analysis. Total cytoplasmic RNA was isolated from MIA PaCa cells by lysis of the cells in isotonic buffer with 0.5% NP-40 in the presence of ribonucleoside vanadyl complex,[15] followed by extraction of the postnuclear fraction with phenol-chloroform and ethanol precipitations. Membrane-containing and membrane-free fractions were prepared[16] from PANC-1 cells, and the RNA from the fractions was isolated as described. Poly (A) + RNA was isolated by oligo (dT) chromatography.[17] The mRNA was run on denaturing formaldehyde gels (18) and then transferred to nitrocellulose filters.[19] The filters were prehybridized and hybridized in a buffer containing 5 × standard saline citrate (SSC), 35% formamide, 5 × Denhardt's,[20] 20 mmol/L of sodium phosphate (pH 7.0), 200 μg/mL yeast RNA, 5 mmol/L of EDTA, and 0.1% sodium dodecyl sulfate (SDS). Hybridization was at 45 °C for 20 hours with 2×10^6 cpm per milliliter of CSF-1 oligonucleotide which had been labeled at the 5′ end with γ-^{32}P ATP by T4 kinase. The 32-base oligonucleotide was derived from amino acids 24 through 34 of the mature CSF-1 sequence.[7] The filters were washed at 45 °C with 5 × SSC–0.1% SDS and 2 × SSC–0.1% SDS. Autoradiography was at −70 °C using Kodak XAR film and DuPont Quanta III intensifier screens.

RESULTS

Human cell lines producing CSF-1. Supernatants of human cell lines were screened for growth activity on murine bone marrow and for macrophage-type CSF-1 by a specific radioreceptor assay that detects both human and murine CSF-1. Table 2 shows that a number of carcinoma lines growing in FCS-supplemented medium constitutively produce large amounts of myeloid growth factors. The supernatants showed two very different titration patterns in promoting DNA synthesis in bone marrow cells (Fig 1). The pattern exemplified by the 5637 bladder carcinoma supernatant (and SK-Hep-1 hepatoma and T-24 bladder carcinoma, not shown) was a low plateau of DNA synthesis, at four to five times background, extending over a wide range (20-fold) of dilutions before titering out. In contrast, supernatants of the pancreatic lines PANC (not shown) and MIA PaCa-2, and the murine L929 fibrosarcoma, induced a much higher plateau level of marrow cell proliferation but tended to titer out faster. These two general patterns suggest that at least two kinds of CSF are being assayed and that different target populations are responding. GCT CSF appears to include a moderate content of CSF-1, as seen by antibody neutralization (Table 1) and titration in the proliferation assay (Fig 1). Pure recombinant interleukin-2 (IL-2), a T lymphocyte growth factor, did not induce significant proliferation in the marrow cells (not shown).

Table 1. Specific Inhibition of CSF-1 in the Proliferation Assay by a Neutralizing Antiserum

Cell Line	Proliferation assay (CSF U/mL)	
	−Ab	+Ab
MIA PaCa CM	2,000	325
MIA PaCa Fr. III	3,358	500
GCT CM	2,329	1,634
5637 CM	1,391	1,479
PANC CM	607	30

CSF, colony stimulating factor = 1, MIA PaCa and PANC, pancreatic carcinoma lines; GCT, Giant Cell Tumor (GIBCO, Grand Island, NY); CM, conditioned medium.

MIA PaCa CM, serum-free purified CSF-1 fraction III, and other CM samples were measured in the proliferation assay as described in the Materials and Methods section. Normal rabbit (−Ab) and specific neutralizing anti-CSF-1 gamma globulin (+Ab) were added at 5% of their serum concentration.

Table 2. Characterization of CSF Produced by Several Human Cell Lines

Cell Line	CSF (U/mL)			
	RRA	Prolif.	BM Colonies	Morphology
MIA PaCa				
10% FCS	3,000	3,700	1,700	44% M, 56% NM
Serum-free (induced)	3,000	ND	3,700	—
Lectin-purified	20,000	20,000	30,700	94% M, 6% NM
GCT	200	2,000	1,500	15% M, 85% NM
5637	100	2,000	1,000	88% NM, 12% N
PANC*	30,000	20,000	10,000	84% M, 16% NM

CSF, colony stimulating factor; RRA, radioreceptor assay; BM, bone marrow; FCS, fetal calf serum; M, macrophage type; NM, mixed type.

MIA PaCa-conditioned media in 10% FCS, serum-free and induced with 10 ng/mL phorbol myristic acetate, or serum-free fraction III (lentil-lectin–Sepharose purified) were tested, as were conditioned media from GCT, 5637, and PANC cell lines. Assays included the CSF-1 specific RRA, mouse bone marrow proliferation assay, and colony assay with cells in individual colonies scored morphologically. The CSF produced by GCT and 5637 that is detected in proliferation and colony assays includes very little CSF-1, seen by the RRA and colony cell morphology.

*Concentrated by ammonium sulfate precipitation.

By specific radioreceptor assay, MIA PaCa and PANC were found to produce high amounts of CSF-1, whereas the other lines were either negative or low producers (Table 2). Selected preparations were tested for bone marrow colony formation, and the colonies were individually picked to determine the hemopoietic lineage of the growing cells. Supernatants of MIA PaCa and PANC, the high CSF-1 producing lines, induced mainly macrophage colonies, whereas other preparations with little or no CSF-1 induced mainly neutrophil or neutrophil-macrophage colonies. The unfractionated conditioned medium of MIA PaCa also contained some GM-CSF[6] that was lost during the purification of CSF-1 (Table 2).

Purified CSF-1 was tested for colony formation on human bone marrow cells. Table 3 shows that MIA PaCa CSF-1 stimulated cluster formation at day 7 and colonies of >50 cells at day 14 of incubation of human mononuclear and nonadherent mononuclear bone marrow cells. CSF-1

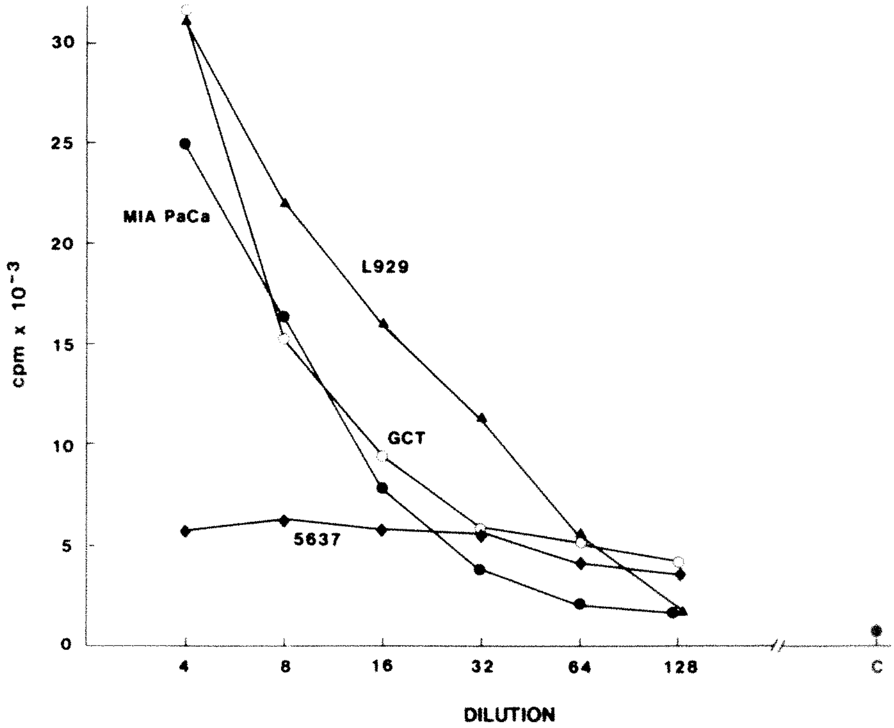

Fig 1. Titration of colony-stimulating factors (CSFs) in the bone marrow proliferation assay. Conditioned media of pancreatic carcinoma line MIA PaCa, GCT, 5637, and L929 cells were diluted and assayed for stimulation of thymidine incorporation as compared with control (C) cultures.

induced fewer colonies in human bone marrow culture than expected, based on titration with murine bone marrow cells. The human colonies consisted mainly of large, disperse cells, as described by Das and colleagues,[2] and were easy to score when the number of input cells was kept to 10^5 cells per plate. Histochemical staining and morphology showed that the colonies were mainly of pure macrophage type, containing large, intensely esterase-positive cells, as compared with mainly neutrophil colony type induced by 5637 conditioned medium (Table 4).

MIA PaCa and PANC cultures were cloned to derive cells capable of high level production of CSF-1. Clones were obtained from both cultures that produced two to three times more CSF-1 than did the parents; low CSF-1 levels were associated with other clones (not shown). However, high-producing clones did not maintain this property on further culture, and subcloning did not generate clones with improved yields. Likewise, no sublines lacking CSF-1 secretion were obtained.

Loss of CSF-1 production in serum-free culture conditions and its reinduction. To produce CSF-1 preparations in low-protein media for purification studies, the effect of serum-free culture was tested. MIA PaCa cells were grown to confluence in medium containing 10% FCS, washed, and recultured in various serum-free formulations. Table 5 shows that CSF-1 production abruptly stopped when no protein or other formulations which included insulin, human albumin, and transferrin were used for culture of the confluent cells. The cells maintained good viability for 3 days in serum-free conditions and resumed CSF-1 production when serum was reintroduced. Similar results were seen with PANC (not shown).

Agents were tested for inducing CSF-1 production in cultures shifted to serum-free conditions at confluence. The results shown are for MIA PaCa, which produced more CSF-1 than did PANC. The strongest inducer was the phorbol ester PMA. Addition of retinoic acid to PMA-induced serum-free cultures further increased production of

Table 3. CSF-1 Stimulation of Colony Formation with Human BM Cells

	LD BM				NALD BM			
	day 7		day 14		day 7		day 14	
	Colonies	Aggregates	Colonies	Aggregates	Colonies	Aggregates	Colonies	Aggregates
Medium	0	0	0	0	0	0	0	0
CSF-1	0	49 ± 3	34 ± 4*	34 ± 5	0	50	42 ± 4*	46 ± 3
CSF-1 1:10	0	0	2 ± 1*	4 ± 0	0	0	2 ± 0*	7 ± 1
5637 CM	56 ± 6	75 ± 6	26 ± 2	32 ± 4	99 ± 5	135 ± 10	42 ± 3	59 ± 2
5637 CM 1:10	7 ± 1	82 ± 9	18 ± 3	28 ± 5	9 ± 1	128 ± 8	31 ± 2	43 ± 2

Abbreviations as in Tables 1 and 2.

Low-density (LD BM) or nonadherent low-density bone marrow cells (NALD BM) (10^5) were plated with 0.1 ml of test sample, and colonies (>50 cells) and total aggregates (colonies plus clusters of 3 to 50 cells) were scored after 7 or 14 days. Purified CSF-1 (fraction II from MIA PaCa cells, 5,000 U/mL by RRA) and CM of the 5637 cell line were tested straight and at 1:10 dilutions.

*Colonies consist of large, disperse cells.

Table 4. Human BM Colony Formation and Type Stimulated by CSF-1

	Colony Formation*		Colony Morphology†		
Sample	Colonies	Aggregates	N	NM	M
Medium	0	4 ± 1			
CSF-1 Fr. II	32 ± 5‡	55 ± 4	9	17	74§
CSF-1 Fr. II 1:10	3 ± 0‡	12 ± 1			
CSF-1 Fr. III	13 ± 4‡	29 ± 1	7	12	81§
CSF-1 Fr. III 1:10	2 ± 1‡	13 + 0			
5637 CM	21 ± 2	26 ± 1	62	14	24
5637 CM 1:10	18 ± 3	23 ± 2			

*Low-density bone marrow cells (10^5) were plated with 0.1-mL test sample, and colonies and total aggregates were scored on day 14 as in Table 3.

†The plates were stained for α-naphthyl acetate esterase and counterstained with Myer's hematoxylin; the colonies were scored for pure neutrophil (N), mixed (NM), or pure macrophage type (M). Purified CSF-1 fractions II and III each contained 10,000 U/mL by RRA.

‡Colonies consist of large, disperse cells.

§Large, mature macrophages with intense esterase staining, some with dendritic-like appearance.

Table 5. Serum-Free Induction of CSF-1 Production by PMA and Retinoic Acid

	CSF-1 (U/mL)			
Media	day 1	day 2	day 3	day 4
Loss of CSF-1 production in serum-free media				
10% FCS	500	1,100	1,450	1,950
2% FCS	—*	—	205	440
0.5% FCS	—	—	—	110
0% FCS	—	—	—	—
0% FCS + PMA	490	1,210	1,870	2,020
HB-104	—	—	—	—
Iscove's	—	—	—	—
Addition	day 1	day 3	day 4	day 5
Induction of serum-free CSF-1 production				
Media	—	—	150	216
3 μmol/L RA	—	—	200	234
10 μmol/L RA	—	—	264	291
30 μmol/L RA	—	75	240	159†
PMA	—	300	333	1,164
3 μmol/L RA + PMA	30	726	717	2,211
10 μmol/L RA + PMA	200	759	1,329	1,953
30 μmol/L RA + PMA	90	801	250†	300†

PMA, phorbol myristic acetate; RA, retinoic acid; other abbreviations as in Tables 1 and 2.

MIA PaCa cells were grown to confluence in T-25 flasks in Dulbecco modified Eagle's medium plus 10% FCS and washed and incubated with FCS or serum-free with 0 or 10 ng/mL of PMA and 0, 3, 10, or 30 μmol/L of RA. Some cultures were reincubated in serum-free HB-104 (Hana Media, Berkeley, Calif), or Iscove's medium (Irvine Scientific, Santa Ana, Calif) plus 1 mg/mL of human albumin and 10 μg/mL of human transferrin. Supernatants were harvested at the times indicated and assayed for CSF-1 by radioreceptor assay.

*<30 U/mL.

†MIA PaCa cells dead.

the growth factor by 50% to 100% (Table 5). Other agents such as lipopolysaccharide, azacytidine, calcium ionophore, merezein, and sodium butyrate, alone or in combinations, were not effective in inducing high levels of CSF-1, nor was a superinduction protocol using cycloheximide and actinomycin D. CSF-1 was detected within 1 day of adding PMA to serum-free cultures, similar to results with washed cultures reincubated with serum-containing medium (Fig 2). Supernatant CSF-1 levels increased through day 4 in induced cultures. PMA added to serum-containing cultures increased CSF-1 production no more than twofold. These results are expressed as CSF-1 per milliliter of culture; because PMA reduced the cell yield slightly, the effect of induction of CSF-1 produced *per cell* was greater than shown.

To investigate whether the uninduced cells in serum-free culture were synthesizing but not secreting CSF-1, confluent MIA PaCa cultures were transferred to serum-free medium and incubated for 2 days with or without inducer. The supernatants were collected, and the cells were lysed by freeze-thawing. The supernatants and cell extracts were then assayed for CSF-1 content. Table 6 shows that CSF-1 recovered from nonsecreting cultures was <10% of that from induced cultures (supernatant and extract), and that in the induced cells >90% of the CSF-1 was secreted.

Northern gel analysis of MIA PaCa and PANC-1 mRNA. As shown in Table 6, incubation of MIA PaCa cells under serum-free conditions without PMA resulted in an almost undetectable level of CSF-1 in the tissue culture supernatants. The CSF-1 present in the cell extracts was comparable to the PMA-induced extracts, however. This indicates that the uninduced cells may be producing a small amount of CSF-1 that is not transported out of the cell. If this is true, the uninduced cells should contain CSF-1 mRNA, and the results shown in Fig 3 directly address this possibility. As seen in lane 4, several species of CSF-1 mRNA in MIA PaCa cells were induced by 48-hour treatment with PMA in serum-free medium, ranging in size from ~1.5 to 4.5 kilobases (kb). Cells induced for 48 hours in the presence of serum (lane 3) showed a similar hybridization pattern, but the signal was much weaker. Cells incubated without serum and PMA (lane 5), still contained appreciable amounts of the large, 4.5-kb mRNA species, but with greatly reduced levels of the smaller mRNAs. Messenger RNA from the membrane fraction of PANC-1 cells, grown in serum containing medium without PMA, synthesized multiple mRNA species (lane 2) similar to those of MIA PaCa cells. CSF-1 mRNA was undetectable in the membrane-free fraction of these cells (lane 1), indicating that the CSF-1 mRNA is located in the endoplasmic reticulum, typical of mRNA's encoding secretory proteins.

DISCUSSION

We describe here the loss and reinduction by PMA and retinoic acid of serum-free production of CSF-1 from MIA PaCa cells. Retinoic acid also appears to have direct stimulating effects on human[21] and murine[22] myeloid progenitor cells. Wu and co-workers reported the spontaneous production of a similar or identical factor from MIA PaCa cells in serum-free medium[6]; the reasons for this difference are unknown.

Our partially purified CSF is CSF-1 by the criteria of the specific radioreceptor assay, correlation of the units of activity measured in that and the mouse bone marrow

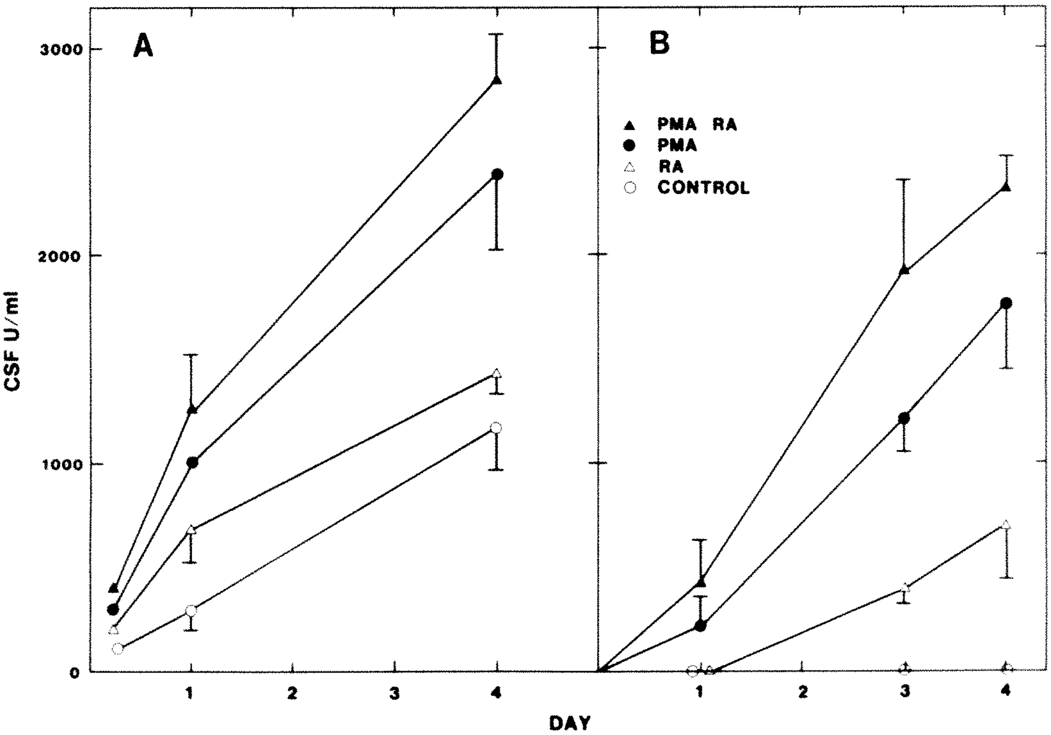

Fig 2. Kinetics of colony-stimulating factor-1 (CSF-1) production by pancreatic carcinoma (MIA PaCa) cells in serum-containing and serum-free media. Cells were grown to confluence in T-25 flasks. The supernatant was replaced with fresh serum-containing (left) or serum-free medium (right) (○), plus 10 μmol/L of retinoic acid (△), plus 10 ng/mL of phorbol myristic acetate (PMA) (●), or plus retinoic acid and PMA (▲). Supernatants were assayed for CSF-1 at 6 hours, 1 day, 3 days, and 4 days (± SD).

proliferation and colony assays, macrophage morphology of the colony cells, and inhibition by specific anti-CSF-1 antibody (Tables 1 and 2). In addition, the MIA PaCa CSF-1 produced mostly diffuse macrophage-type colonies in human bone marrow cultures, as described by Das and colleagues for the apparently identical urinary molecule. However, much more CSF-1 was required for colony formation on human bone marrow cells as compared with murine bone marrow cells (Tables 3 and 4).

Waheed and Shadduck[23] and Motoyoshi and co-workers[24] reported that human urinary CSF-1–like factors were inactive on total or macrophage-depleted human bone marrow cells, respectively, although they were macrophage-type CSFs when tested on murine cells. In assays of Wu and co-workers,[25] MIA PaCa CSF-1 stimulated human bone marrow colony formation at 5% of that seen with murine cells at day 7 of culture, and at 34% by day 13 of culture. We find that CSF-1 colonies are easily detected at day 14 of human bone marrow culture with or without depletion of adherent cells, but are seldom seen at day 7. Differences in assay and scoring methods may explain these discrepancies. The diffuse nature of the human colonies may make their recognition difficult when $>10^5$ cells are plated. The carbonyl-iron method of macrophage depletion used by Motoyoshi and co-workers[24] may remove some of the CSF-1 responsive progenitors. Finally, the authors of these articles may not have tested high enough concentrations of CSF-1, since low levels are less active on human targets than on murine cells.

Table 6. Secreted and Intracellular CSF-1 in Serum-Free Cultures

		CSF-1 (U)			
Culture	Inducer	Supernatant	Cell Extract	Total	(%) Secreted
1	None	<50	796	733	0
		<50	671		
2	PMA	6,300	375	6,768	93
		6,300	561		
3	PMA/RA	18,270	1,845	20,115	91

Abbreviations as in Tables 1, 2, and 5.

MIA PaCa cells were grown to confluence in 10% FCS in T-75 flasks, washed and cultured serum-free with 0 or 50 ng/mL of PMA and 10 μmol/L of RA where shown. After 2 days of incubation, the supernatant was collected, and the cells were freeze-thawed to obtain an extract. CSF-1 was quantitated by the radioreceptor assay.

Induced serum-free production of CSF-1 has aided its purification to homogeneity,[14a] amino acid sequencing, and molecular cloning of its complementary DNA (cDNA).[7] The protein shows extensive homology with murine L cell CSF-1,[7,26] with 75% identical amino acids spanning the residues determined so far in the murine molecule. The protein chemistry and molecular cloning studies confirm the hypothesis that CSF-1 is a homodimer[2,3] and suggest that it is encoded by a single gene.[7]

Although CSF-1 appears to be encoded by a single-copy gene, multiple species of CSF-1 mRNA are expressed in PMA-induced MIA PaCa and normally growing PANC-1

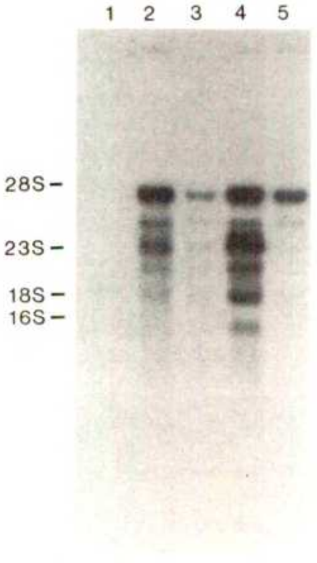

Fig 3. Northern gel analysis of pancreatic carcinoma lines MIA PaCa and PANC-1 mRNA. Lane 1, mRNA from membrane-free fraction of PANC-1 cells; lane 2, mRNA from membrane fraction of PANC-1 cells; lane 3, mRNA from 48-hour phorbol myristic acetate (PMA)–induced MIA PaCa cells incubated with serum; lane 4, mRNA from 48-hour PMA-induced MIA PaCa cells incubated serum-free; lane 5, mRNA from 48-hour MIA PaCa cells incubated serum free. Ten micrograms of each mRNA sample was electrophoresed, blotted, and probed with a 32-base CSF-1 specific complementary oligonucleotide as described in the Materials and Methods section. The MW markers are mammalian and *E. coli* ribosomal RNAs.

(Fig 3) and MIA PaCa cells (not shown). In the cDNA cloning studies,[7] we found that some of the mRNAs contain part of an intron sequence and may not be biologically functional. It is still uncertain whether each size class of CSF-1 mRNA contains biologically active and/or inactive mRNAs. It is intriguing, however, that the MIA PaCa cells incubated in the absence of serum and PMA, while not secreting measurable levels of CSF-1 (Table 6), still have detectable levels of CSF-1 protein in the cell extracts and a large (4.5 kb) mRNA species in the cytoplasm. Among many possibilities, this result is consistent with the hypothesis that removal of the serum from the MIA PaCa tissue culture medium does not block CSF-1 mRNA synthesis per se, but seems to hinder some step(s) in the pathway of mRNA processing and/or CSF-1 protein secretion. In support of this hypothesis, previous studies[27,28] have indicated that the CSF-1 protein may exist in two forms; a secretory and a cell surface form. The CSF-1 cDNA predicts the translation of a precursor protein with a C-terminal transmembrane hydrophobic region and triple Arg anchor site.[7] The large mRNA species may represent the coding sequence for the membrane bound form of CSF-1. Precedence for this type of phenomenon is found in other systems such as the immunoglobulins.[29] Experiments are in progress to determine the origin and significance of the multiple CSF-1 mRNA species and whether there is indeed a membrane bound form of CSF-1.

CSF-1 has stimulating effects on the mature monocyte and macrophage in addition to its growth and differentiation activity. Murine CSF-1 promotes the production of prostaglandin E,[30] IL-1,[31] plasminogen activator,[32,33] and interferon,[34] and acidic isoferritins.[35] It also stimulates tumor cytostasis,[36] peroxide formation,[37] and probably uptake and intracellular killing of *Candida*[38] by macrophages. Motoyoshi and colleagues[24] showed that a human urinary CSF-1-like factor induced myeloid colony formation in human marrow cultures in the presence but not the absence of adherent cells, suggesting that marrow macrophages were stimulated to produce a different type of CSF. Metcalf and Nicola described the production of G-CSF by murine peritoneal macrophages that had been stimulated with CSF-1.[39] We found that MIA PaCa CSF-1 promoted the production of interferon, tumor necrosis factor, and a myeloid CSF by peripheral blood monocytes.[40] The LPS content of CSF-1 preparations is very low and does not appear to have a role in the biologic properties of the macrophage growth factor.[40]

With the cloning of a CSF-1 cDNA, large quantities of purified protein can be produced for possible therapeutic use. In patients with bone marrow depression occurring naturally or due to cancer chemotherapy or irradiation, CSF-1 may be used therapeutically to stimulate the recovery of monocytes and indirectly, by stimulating endogenous myeloid CSF production, the recovery of neutrophils. In addition, CSF-1 may have direct promoting effects on mononuclear phagocytes to improve the body's resistance to infectious diseases and to stimulate the macrophages within tumors to destroy the neoplastic cells.

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Selenium-Dependent Glutathione Peroxidase Protein and Activity: Immunological Investigations on Cellular and Plasma Enzymes

By Kazuhiko Takahashi and Harvey J. Cohen

Selenium-deficient humans and animals are known to be deficient in glutathione peroxidase (GSHPx) activity in their cells and plasma. To determine the relationship between enzyme activity and protein content, the enzyme was purified from human erythrocytes, and polyclonal antibodies were made against the purified protein in rabbits. These antibodies were found to be monospecific, noninhibitory, and capable of precipitating the enzymatic activity. All the GSHPx activity in erythrocytes and almost all the activity in neutrophils and platelets was precipitated by these antibodies. None of the plasma enzyme was precipitated by these antibodies, indicating that the plasma enzyme activity was attributable to a different selenium dependent protein moiety. Utilizing a radioimmunoassay, we were able to determine that there was a direct relationship between GSHPx activity and protein content in the erythrocytes of both normal and selenium-deficient individuals, and a similar relationship between control and selenium-deficient rat erythrocytes and liver cells. Thus, the ability to examine GSHPx as a protein resulted in two new observations concerning the selenium-dependent GSHPx. The first is that the plasma enzyme is antigenically distinct from the erythrocyte enzyme, and the second is that in the absence of selenium, there is a concomitant decrease in GSHPx protein.

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GLUTATHIONE peroxidase (GSHPx) plays an important role in the detoxification of hydrogen peroxide, organic hydroperoxides, or lipid peroxides.[1-3] Selenium (Se)-deficient animals and humans have decreased GSHPx activity.[4-7] Because Se in the form of selenocysteine is present in the active site of this enzyme,[8] it is not surprising that Se deficiency results in a decrease in enzymatic activity. However, it was not known whether the absence of Se could affect the GSHPx protein or whether apo-enzyme was present. To determine if there is a direct relationship between GSHPx activity and protein in Se deficiency, or if apo-protein exists, we purified the enzyme from erythrocytes and developed polyclonal, monospecific antibodies against the enzyme. Utilizing these antibodies in a radioimmunoassay, we were able to correlate protein content with enzymatic activity. We were also able to show that these antibodies cross-reacted with rat GSHPx and that Se-deficient rat liver as well as rat erythrocytes had a concomitant decrease in GSHPx protein content and activity. Finally, using this antibody, we were able to determine that the plasma GSHPx activity, although Se dependent, is not due to the same antigenic material as that found in erythrocytes, neutrophils, or platelets.

MATERIALS AND METHODS

Materials. Reduced glutathione (GSH), glutathione reductase (type IV, from baker's yeast), bovine serum albumin (essentially fatty acid and globulin free), NADPH, Triton X-100, and t-butyl hydroperoxide (t-BuOOH) were purchased from Sigma Chemical Company, St Louis; Dextran T500, Ficoll-Paque, DEAE-Sephadex A-25, Sephadex G-200, and Sepharose 2B from Pharmacia Fine Chemicals, Piscataway, NJ; diethylaminoethanol (DEAE)-cellulose DE-52 and CM-cellulose CM-52 from Whatman, Clifton, NJ; hydrogen peroxide from Fisher Scientific, Pittsburgh; Bolton-Hunter reagent from New England Nuclear, Boston; and Freund's complete adjuvant, rabbit IgG and goat anti-rabbit IgG antibody from Calbiochem, La Jolla, Calif. Other chemicals were of analytical grade and were obtained commercially.

Sources of cells. Samples of erythrocytes were obtained from normal and Se-deficient individuals as previously described.[9] The erythrocytes from one of these individuals were used during the course of Se repletion.[9] Control and deficient rats were fed diets as previously described[10] for >3 months prior to analyses.

GSHPx activity. GSHPx activity using t-butyl hydroperoxide as a substrate was assayed by an adaptation of the method of Beutler.[11] Both sample and reference cuvettes contained 0.1 mol/L of Tris-HCl, pH 8.0, 0.5 mmol/L of EDTA, 0.2 mmol/L of NADPH, 1 U of glutathione reductase, 2 mmol/L of GSH, and the appropriate amount of enzyme in 1 mL. The oxidation of NADPH by t-BuOOH (70 μmol/L), added to the sample cuvette only, was followed spectrophotometrically at 340 nm at 37 °C. An additional blank containing all components except the enzyme was determined to correct for nonenzymatic oxidation of GSH and NADPH by t-BuOOH.

GSHPx activity using hydrogen peroxide as a substrate was assayed as follows. Both sample and reference cuvettes contained 0.1 mol/L of sodium phosphate buffer, pH 7.0, 0.5 mmol/L of EDTA, 0.2 mmol/L of NADPH, 1 U of glutathione reductase, 1 mmol/L of NaN_3, 2 mmol/L of GSH, and the appropriate amount of enzyme in 1 mL. The oxidation of NADPH by hydrogen peroxide (15 μmol/L), added to the sample cuvette only, was followed at 340 nm at 37 °C. An additional blank was measured as described above. One unit of enzyme activity is defined as the oxidation of one nmol of NADPH per minute.

Cell extracts and plasma preparation. To prepare plasma and erythrocytes, human or rat blood collected in heparin was centrifuged in a Triac Combination centrifuge for five minutes. The plasma was removed and assayed for GSHPx activity. Erythrocytes were washed three times in 0.154 mol/L of NaCl. To 0.1 mL of the washed packed erythrocytes, 1.9 mL of 1 mmol/L of potassium phosphate buffer, pH 7.5, was added. The resulting hemolysate was centrifuged at 14,000 g for 10 minutes to remove the erythrocyte membranes. The clarified hemolysate was assayed for hemoglobin and GSHPx activity.

Human granulocytes (PMNs) were prepared by dextran and Ficoll-Paque sedimentation from blood collected in acid-citrate-dextrose as described previously.[12] Rat PMNs were obtained from

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their peritoneal cavities 18 hours after the intraperitoneal injection of casein as previously described.[13] PMNs at a concentration of 1 × 10^8 cells/mL, were suspended in 0.34 mol/L of sucrose, 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.1% Triton X-100, and placed in a sonicator bath for six to eight seconds. The lysates were centrifuged at 70 g for five minutes at 4 °C. The supernatant was centrifuged at 20,000 g for 20 minutes at 4 °C. The resulting cytosolic solutions were assayed for GSHPx activity.

Human platelets were prepared from platelet-rich plasma by gel filtration on Sepharose 2B as described previously.[14] Platelets at a concentration of 3.5 × 10^8 cells/mL, were suspended in 0.34 mol/L of sucrose, 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.1% Triton X-100, and placed in a sonicator bath for six to eight seconds. The lysates were centrifuged at 20,000 g for 20 minutes at 4 °C. The resulting cytosolic solutions were assayed for GSHPx activity.

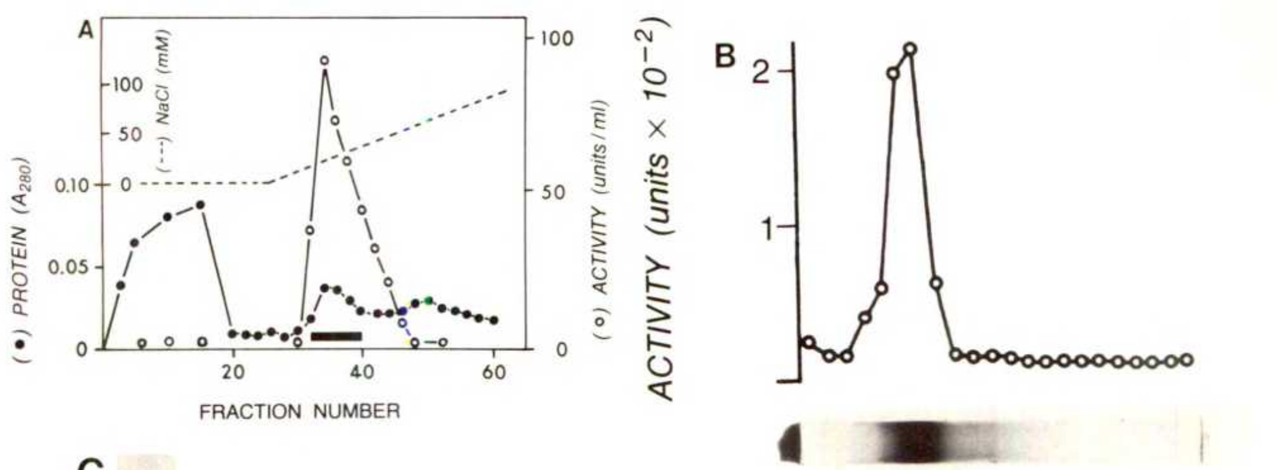
Human liver obtained at autopsy, or rat liver, was rinsed with cold 0.154 mol/L of NaCl, blotted, weighed, and homogenized in 9 vol of 0.25 mol/L of sucrose, 0.02 mol/L of Tris-HCl, pH 7.4, in a glass Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 600 g for 10 minutes to remove whole cells and debris. This supernatant was centrifuged at 12,000 g for 20 minutes at 4 °C to pellet the mitochondria. The resulting cytosolic solutions were assayed for GSHPx activity.

Purification of human erythrocyte GSHPx. GSHPx was purified from human erythrocytes according to the methods of Awasthi and colleagues[15] including ammonium sulfate fractionation, CM-cellulose CM-52, DEAE-cellulose DE-52, Sephadex G-200, and DEAE-Sephadex A-25 column chromatography.

Gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) (6% acrylamide gel) was performed by the method of Davis.[16] Sodium dodecyl sulfate (SDS)-PAGE (10% acrylamide) was performed by the method of Laemmli.[17] After electrophoresis, proteins were stained as described previously[18] or eluted from gels.

Immunization and isolation of antibody. One hundred and fifty micrograms of purified GSHPx was mixed with Freund's complete adjuvant and injected intramuscularly in four sites on the back of a New Zealand white rabbit. This procedure was repeated weekly for 4 weeks. Serum was collected 5 weeks after the first immunization. Because rabbit serum itself contains GSHPx activity, the IgG fraction was purified free from GSHPx activity using ammonium sulfate precipitation followed by DEAE-cellulose DE-52 chromatography.

Radioimmunoassay. Radioimmunoassays were performed using the double antibody technique of Roberts and Parker[19] with a slight modification. GSHPx purified from human erythrocytes was radio-iodinated using the Bolton-Hunter reaction.[20] Unknown sam-



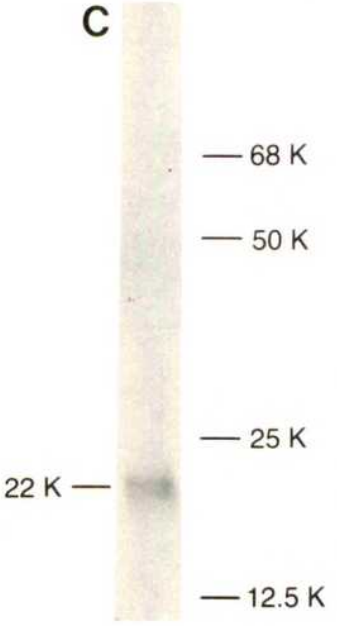


Fig 1. Purity of isolated glutathione peroxidase (GSHPx). (A) Diethylaminoethanol (DEAE)-Sephadex A-25 column chromatography as the final purification step for GSHPx. Partially purified fractions of GSHPx dialyzed against 5 mmol/L of potassium phosphate buffer, pH 6.0, containing 0.7 mmol/L of β-mercaptoethanol were applied to a 1.5 × 3.0 cm column of DEAE-Sephadex A-25 equilibrated with the same buffer. The column was washed with this buffer, and the proteins were subsequently eluted with a linear gradient of 0 to 100 mmol/L of NaCl. Protein was detected by measuring absorbance at 280 nm, and GSHPx activity was measured as described in the Materials and Methods section. The fractions under the bar were concentrated by the use of Amicon Centriflo. (B) Polyacrylamide gel electrophoresis (PAGE) of purified GSHPx. Twenty micrograms of purified enzyme were applied to each gel. After electrophoresis, protein was stained by the method of Burges[18] and GSHPx activity was measured in 2 mm slices of the gel. (C) Sodium dodecyl sulfate (SDS)-PAGE of purified GSHPx. Twenty micrograms of purified enzyme was treated with 1% SDS and 1% β-mercaptoethanol and applied to the gels. After electrophoresis, protein was stained as described above. The mol wt was estimated using bovine serum albumin, with the heavy and light chains of human IgG and cytochrome as standards.

ples and standard samples (purified GSHPx) were incubated with 10 μL of rabbit anti-GSHPx IgG (0.65 mg/mL of the isolated IgG, with 1 mg/mL carrier rabbit normal IgG) for 15 minutes at 25 °C followed by an additional 60-minute incubation at 25 °C with ~1,500 cpm of ^{125}I-GSHPx (20 μL). The reaction mixtures contained 50 mmol/L of Tris-HCl, pH 8.0, 2.5 mmol/L of EDTA, 0.2% NaN$_3$ and 0.2% bovine serum albumin in a total volume of 160 μL. After incubation, 40 μL of goat anti-rabbit IgG antibody was added at equivalence and incubated for 15 minutes at 25 °C. The mixtures were then centrifuged at 10,000 g for 5 minutes at 4 °C, and the radioactivity of resulting immune precipitates was determined. The amount of GSHPx in unknown samples was calculated from a standard curve obtained with purified GSHPx (1 to 100 ng).

Protein and hemoglobin determination. The protein concentration was measured by the method of Lowry et al,[21] using bovine serum albumin as a reference protein. Hemoglobin concentration was determined with Drabkin's reagent.

RESULTS

Purity of human erythrocyte GSHPx. Using the method previously described by Awasthi and colleagues,[15] we purified human erythrocyte GSHPx to homogeneity. Unlike previously described results, we could not find two peaks of GSHPx activity when gel filtration on Sephadex G-200 was performed and found only a single peak of activity during the final purification step. The elution pattern of DEAE-Sephadex A-25 column chromatography is shown in Fig 1A. PAGE revealed a single protein stained band, shown in Fig 1B. Slicing the polyacrylamide gels into 2-mm slices, followed by elution of the slices into buffer resulted in the recovery of a single peak of GSHPx activity, which conformed to the protein peak as shown in Fig 1B. SDS-PAGE of the purified enzyme showed a single band with mol wt of 22,000, as shown in Fig 1C. This is similar to the major GSHPx protein that was previously described.[15] The specific activity of the purified enzyme was 224 units/mg.

Properties of anti-GSHPx antibody. Rabbit antibody against human erythrocyte GSHPx was prepared as described in the Methods section. Because rabbit serum contains GSHPx activity, the IgG fraction of rabbit serum was separated from GSHPx using ammonium sulfate precipitation followed by DEAE-cellulose DE-52 column chromatography. The resulting IgG fraction contained no GSHPx activity. Figure 2 shows a result of the Ouchterlony analysis of the antibody preparation. A single precipitin line between the antibody preparation and purified GSHPx was found. An identical single precipitin line was also found when the antibody reacted with the initial partially purified fractions during purification of GSHPx, and a zone of identity was present between the lines formed with the crude fractions and the purified enzyme, indicating that no major modifications of the enzyme occurred during purification. Thus, we had monospecific polyclonal antibodies against erythrocyte GSHPx. To determine the ability of these antibodies to inhibit enzymatic activity, we incubated various amounts of antibody with the purified enzyme and either directly measured enzyme activity or measured enzyme activity of the supernatant after centrifugation of the mixture at 100,000 g. As shown in Fig 3, the antibody did not inhibit GSHPx activity, but did precipitate the activity in a dose-dependent manner. Control rabbit IgG did not precipitate the enzymatic activity (data not shown). Thus, we had monospecific, polyclonal, precipitating antibodies that did not inhibit enzyme activity and therefore were not directed against the active site of the enzyme.

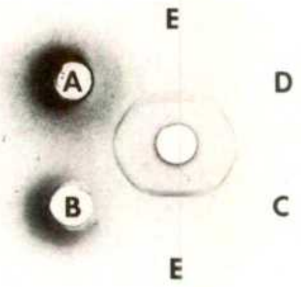


Fig 2. Ouchterlony immunodiffusion analysis of anti-glutathione peroxidase (GSHPx) IgG. The center well contained anti-GSHPx IgG. Wells A, B, C, D, and E contained the ammonium sulfate fractionation, CM-cellulose CM-52, diethylaminoethanol (DEAE)-cellulose DE-52, Sephadex G-200, and DEAE-Sephadex A-25 fractions, respectively. The diffusions were performed in 1% agar. The gels were washed with 1 mol/L of NaCl to remove the unreacted protein and were stained for protein using 0.02% Coomasie brilliant blue R-250.

Reactivity of antibody with cell lysates. Figure 3 also shows the result of incubating increasing amounts of this antibody preparation with erythrocyte hemolysates followed by centrifugation on the GSPHx activity present in the supernatant. There was a decrease in supernatant GSHPx activity with increasing amounts of antibody. At the highest concentration of antibody used, no GSHPx activity was demonstrable in the resulting supernatant. This result confirms our previous results with Se-deficient erythrocytes, indicating that all of the GSHPx activity present in human erythrocytes is due to this Se-dependent GSHPx protein.[9] We then examined other human cells for reactivity of their GSHPx protein with this antibody. Table 1 shows that

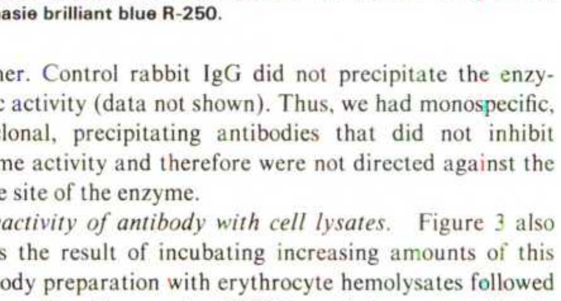


Fig 3. Precipitation of glutathione peroxidase (GSHPx) by rabbit anti-GSHPx IgG. Samples containing 0.03 U of purified GSHPx (●) or 0.03 U of a 1:20 hemolysate from human erythrocytes (□) were incubated at 25 °C for 60 minutes with rabbit anti-GSHPx IgG. The mixtures were centrifuged at 100,000 g for 30 minutes at 4 °C, and the resulting supernatants were assayed for GSHPx activity. An additional sample of the purified enzyme was also incubated with anti-GSHPx IgG as described above and then assayed for enzyme activity without centrifugation (○).

Table 1. Precipitation of Human Cellular GSHPx Activity by Rabbit Anti-GSHPx IgG

		GSPHx Activity (× 10^{-3} U)		
Preparation	Substrate	− Antibody	+ Antibody	Control (%)
Neutrophils	t-BuOOH	11.6	1.45	12.5
Platelets	t-BuOOH	8.09	0.26	3.2
Liver cytosol	t-BuOOH	2.91	0.90	30.9
Liver cytosol	H_2O_2	1.58	<0.1	<6

Samples were incubated at 25 °C for 60 minutes either with or without rabbit anti-GSHPx IgG (225 µg). The mixtures were centrifuged at 100,000 g for 30 minutes at 4 °C, and the resulting supernatants were assayed for GSHPx activity. Results are the average of duplicate determinations.

~87% of the PMN and 97% of the platelet GSHPx activity was precipitated with this antibody. Of note is that ~70% of the human liver cytosol GSHPx activity was precipitated with this antibody when t-BuOOH was used as a substrate. However, when hydrogen peroxide was used as a substrate, >94% of the GSHPx activity in liver cytosol was precipitated. In additional studies, we determined that this antibody could also precipitate GSHPx activity from rat erythrocytes, PMNs and liver cytosol. This antibody preparation could not precipitate rabbit erythrocyte GSHPx activity.

In previous studies, we showed that although plasma GSHPx and erythrocyte GSHPx activities were markedly diminished in Se-deficient individuals, the plasma enzyme activity did not come from the erythrocyte, since the plasma enzyme activity returned to normal within 2 to 3 weeks after Se supplementation was initiated, prior to any substantial increase in erythrocyte enzyme activity.[9] We therefore examined the ability of the anti-erythrocyte GSHPx antibody to precipitate the plasma enzyme. As can be seen in Table 2, the addition of purified erythrocyte GSHPx to plasma and the subsequent addition of antibody followed by centrifugation resulted in the precipitation of only that amount of enzyme activity contributed by erythrocyte GSHPx. Thus, there appear to be antigenic differences between the erythrocyte and plasma enzymes. The antibody preparation was also unable to precipitate rat plasma GSHPx activity.

Relationship of enzyme activity to protein content. Using a radioimmunoassay as described in the Methods section, we examined the relationship between protein content and GSHPx activity in erythrocytes from normal individuals and Se-deficient individuals, including one individual studied during repletion with Se. As shown in Fig 4, there is a direct relationship between GSHPx protein and GSHPx activity in the hemolysates from normal erythrocytes. The average specific activity of GSHPx from normal erythrocytes as determined from this radioimmunoassay is 0.215 ± 0.023 U/µg of GSHPx. This value correlates well with the specific activity of the purified enzyme from human erythrocytes (0.224 U/µg). The hemolysates from Se-deficient individuals displayed a similar relationship of enzyme activity and protein content. In addition, during the course of repletion, there was again a correspondence of 1:1 between GSHPx activity and protein content. Thus, in erythrocytes from Se-deficient individuals, there is not only an absence of enzyme activity but a concomitant decrease in protein content. The correlation coefficient for this linear relationship was found to be 0.91 (n = 15). We found the same relationship between protein content and enzyme activity in hemolysates from both control and Se deficient rat erythrocytes, as shown in Table 3. Because erythrocytes are not capable of synthesizing protein, we examined the relationship between GSHPx activity and protein content in rat liver from both control and Se-deficient rats. Table 3 shows that, as for the human erythrocyte hemolysate, Se-deficient rat erythrocytes and liver cytosol have similar amounts of GSHPx activity and GSHPx protein content. In our assay system, the specific activity of rat GSHPx is 2.7-fold greater than that of the human erythrocyte GSHPx. Using the same radioimmunoassay, we found <1% of antigenically detectable GSHPx protein in human plasma, as would be expected for the amount of enzyme activity present. This supports the results shown in Table 2.

Table 2. Precipitation of Plasma GSHPx Activity by Rabbit Anti-GSHPx IgG

	GSHPx Activity (× 10^{-3} U)	
Preparation	− Antibody	+ Antibody
1× purified enzyme	7.10	<0.10
2× purified enzyme	14.20	<0.10
Plasma	5.00	5.20
Plasma + 1× purified enzyme	12.90	5.47

Samples were incubated at 25 °C for 60 minutes either alone or with rabbit anti-GSHPx IgG (225 µg). The mixtures were centrifuged at 100,000 g for 30 minutes at 4 °C, and the resulting supernatants were assayed for GSHPx activity. Results are the average of duplicate determinations.

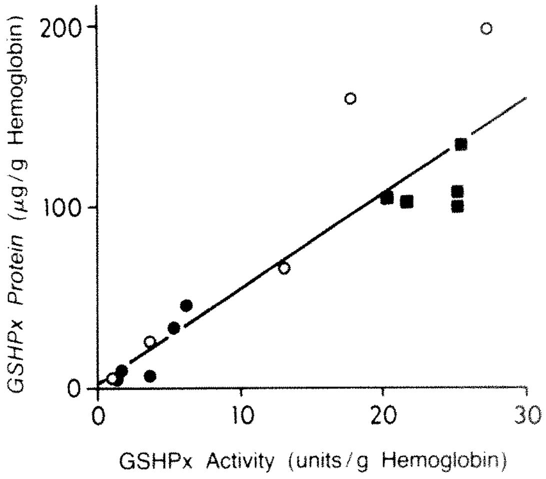


Fig 4. Relationship between GSHPx activity and protein in human erythrocytes. Samples were obtained from five normal individuals (■), five Se-deficient individuals (●), and a Se-deficient individual during the course of Se repletion (○) as described previously.[9] The hemolysates were assayed for hemoglobin content, GSHPx activity, and protein as described in the Materials and Methods section.

DISCUSSION

GSHPx, as found in mammalian cells, is a Se-dependent enzyme.[22-24] The mechanism of action of this enzyme

Table 3. Activity and Protein of Erythrocyte Hemolysate and Liver Cytosol GSHPx From Control and Se-Deficient Rats

Enzyme Source	GSHPx Activity (% Control)	GSHPx Protein (% Control)	Specific Activity (U/μg)
Control erythrocytes	235*	425†	0.553
Se-deficient erythrocytes	13.8* (5.9)	25.9† (6.1)	0.533
Control liver cytosol	2.96‡	4.69§	0.631
Se-deficient liver cytosol	0.046‡ (1.6)	0.080§ (1.7)	0.575

Samples were obtained from control and Se deficient rats as described previously[10] and assayed for hemoglobin (or protein) content, GSHPx activity, and GSHPx protein as described in the Materials and Methods section.

*Hemoglobin (U/g).

†Hemoglobin (μg/g).

‡Protein (U/mg).

§Protein (μg/mg).

requires the presence of selenocysteine in the active site.[8] Both experimental and clinical Se deficiency result in decreased enzyme activity.[4-7] Without the ability to measure specific protein content unequivocally, it was not possible to distinguish between an inactive enzyme and the absence of the protein. To measure protein content, we required the development of polyclonal, monospecific antibodies that did not inhibit enzyme activity. Polyclonal antibodies were required rather than monoclonal antibodies in case the monoclonal antibodies were directed against a determinant not found in the Se-deficient protein. Monospecificity was required to be able to measure only this protein. Because Se is part of the active site of GSHPx, it was important that the antibodies were not directed against the active site that may be altered in the absence of Se. The antibodies made in the rabbit against human erythrocyte GSHPx satisfied all the above criteria and therefore were used to examine the nature of the GSHPx protein.

The observation that this antibody preparation could precipitate all the GSHPx activity from erythrocytes and most of the enzyme activity from PMN and platelets is evidence that the Se GSHPx enzyme is the major GSHPx activity in these cells. Because the radioimmunoassay does not require active enzyme in cell preparations, and because there is a direct relationship between enzyme activity and protein content in the hemolysates from normal individuals, this radioimmunoassay can be used to measure GSHPx in samples that have been stored for prolonged periods of time.

The ability of this antibody to react with rat cellular GSHPx demonstrates that the rat GSHPx protein is immunologically similar to the human protein. The inability of the rabbit antibody to react with human or rat plasma GSHPx was an unexpected and intriguing finding. We had previously suspected that the plasma enzyme activity was not related to erythrocyte activity,[9] but until these investigations were performed, there was no evidence that it might have a different antigenic structure. A logical source for the plasma enzyme may be the liver. As demonstrated above, the anti-GSHPx antibody precipitated ~70% of the activity in human liver cytosol when assayed with t-BuOOH and almost all the enzyme activity when assayed with hydrogen peroxide. Because the plasma enzyme has hydrogen peroxide-dependent activity[25] and because ~30% of human liver cytosol GSHPx assayed by t-BuOOH is not from a Se-dependent enzyme but from glutathione S-transferase,[26] it does not appear that the liver cytosolic enzyme is the direct source of the plasma GSHPx protein. It is still possible that liver cytosol enzyme is modified for transport prior to secretion in the plasma and that this modified protein may not react with the antibody against native GSHPx. Zakowski and Tappel described a liver mitochondrial Se-dependent GSHPx with different properties from the cytosol enzyme.[27] Although unlikely, it is possible that the plasma enzyme is derived from this form of liver GSHPx. We are currently investigating the nature of the plasma enzyme.

The absence of GSHPx protein in Se-deficient erythrocytes suggests that there may be a relationship between Se and synthesis of GSHPx protein. These results are similar to those obtained using Se-deficient rats.[28] Sunde and Evenson have found that the selenocysteine of rat liver GSHPx comes from serine and is therefore due to either a cotranslational or posttranslational modification.[29] Hawkes and colleagues, however, have demonstrated evidence for a specific selenocysteine transfer RNA in rat liver.[30] Thus, it is possible that the absence of Se results in a deficiency of selenocysteine, which might also result in the inability to translate the message for GSHPx. Interpretation of the studies in erythrocytes are complicated by the inability of these cells to synthesize protein. Thus, a deficiency of GSHPx protein in these cells may be due to instability of an abnormal protein made in the absence of Se. The studies of rat liver GSHPx described here are consistent with the view that even cells capable of synthesizing protein may not synthesize GSHPx in the absence of Se.

We have recently examined the relationship between GSHPx protein and activity in the human promyelocytic cell line HL-60 grown in the absence of Se and then supplemented with sodium selenate. We found a direct relationship between GSHPx protein content and enzymatic activity.[31] Thus, in a human cell line capable of synthesizing protein and GSHPx specifically,[32] there was an absence of the protein in the Se-deficient state. The studies of rat liver described here and the previously described studies on HL-60 cells are consistent with the view that even cells capable of synthesizing protein may not synthesize GSHPx in the absence of Se. It is still possible that an abnormal protein is made and very rapidly degraded.

The ability to study GSHPx as a protein in addition to studying it as an enzyme, using polyclonal, monospecific, noninhibitory precipitating antibodies, has resulted in two important new pieces of information concerning Se-dependent GSHPx. First, the plasma GSHPx enzyme is antigenically different from the erythrocyte GSHPx enzyme; second, in the absence of Se, there is concomitant decrease in GSHPx protein. The biochemistry and molecular biology of these phenomena await further investigations.

ACKNOWLEDGMENT

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Italian Type of Deletional Hereditary Persistence of Fetal Hemoglobin

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We report a new type of deletion of the β globin gene cluster in the Italian population that confers a phenotype of hereditary persistence of fetal hemoglobin (HPFH) to the carriers. This deletion begins ~5 kilobases (kb) 5′ to the δ globin gene and ends ~30 kb 3′ to the β globin gene, in close proximity to the 3′ end of an Indian HPFH. In all four previously described HPFH, a repetitive Alu I region 5′ to the δ globin gene is largely or completely deleted; the 5′ end of the new HPFH is consistent with this common feature. In addition, the finding that Italian and Indian HPFHs, as reported for other groups of deletions, have very close 3′ ends, strengthens the idea that common mechanisms may operate in generating these deletions. Finally, we show that, in spite of similar 5′ breakpoints, the deletion of Spanish $\delta\beta^{\circ}$-thalassemia is at least 8 kb longer than that of Negro HPFH type I, thus ruling out the hypothesis that the overall extent of the deletion might influence the level of γ globin chain synthesis.

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HEREDITARY persistence of fetal hemoglobin (HPFH) and $\delta\beta$-thalassemia are inherited disorders characterized by the persistent synthesis of fetal hemoglobin ($\alpha_2\gamma_2$) during adult life. Patients with HPFH show a higher level of γ chain synthesis, an almost balanced ratio of α/non-α globin chain synthesis, and only slight red cell abnormalities; on the contrary, $\delta\beta$-thalassemia patients have a lower level of γ globin synthesis and a more pronounced thalassemic phenotype.[1] In the last years, these rare conditions have been extensively investigated at the molecular level to provide better understanding of the physiology of the hemoglobin switch.

A large group of HPFH and $\delta\beta$-thalassemias is characterized by extensive deletions in the β-like globin gene cluster.[2-19] Molecular studies of these conditions have led to two main groups of hypotheses to explain the different level of γ chain synthesis observed in these syndromes. First, γ gene expression may be influenced by the overall extent of the deletion; larger deletions may generate HPFH, and smaller ones may generate $\delta\beta$-thalassemia by disrupting the normal chromatin structure in different ways. Second, a deletion may be effective either by removing DNA regions or by bringing new sequences to the globin gene region.[20] The loss or addition of specific sequences in the non-α globin gene cluster may be relevant to the level of expression of γ globin genes. For example, in Sicilian $\delta\beta$-thalassemia, ~6 kilobase (kb) pairs from the β globin gene and 3′ to it are additionally lost in comparison with the deletion causing Hb Lepore, resulting in higher expressions of γ globin genes[3-5,11-12]; on the other hand, a small region containing a pair of inverted Alu I repeats located 5′ to the δ globin gene area is consistently deleted in HPFH[10] but not in $G_\gamma A_\gamma$ $\delta\beta^{\circ}$-thalassemia (Spanish type).[9] These data could suggest that loss of specific DNA regions either 3′ or 5′ to the β globin cluster may influence the level of γ globin gene expression; on the other hand, these same deletions have the potential of bringing "enhancer" sequences, originally situated at some distance, in proximity to the γ globin genes.[21] We have identified a new deletion generating HPFH in three clearly unrelated Italian families. The 5′ endpoint of this deletion maps in close proximity to the Alu I region 5′ to the δ globin gene, where two different deletions causing Negro HPFH type I[10] and Spanish $\delta\beta^{\circ}$-thalassemia[9] begin. On the contrary, the 3′ endpoint of the new deletion is closer to the β globin gene than are those observed in the above two conditions and is very similar to that observed in an Indian HPFH.[22]

MATERIALS AND METHODS

Nine subjects belonging to three different families from Southern Italy were studied. Hematological data and RBC indices were obtained by standard procedures. Hb A_2 levels, Hb F percentage, globin chain synthesis, and the relative proportion of G_γ and A_γ chains in the Hb F were determined as previously described.[23] DNA was obtained from subjects A I-1, B I-1, and C II-2 (see Table 1). DNA preparation and digestion, electrophoretic separation of DNA fragments, transfer to nitrocellulose filters, hybridization, and washing procedures were as previously described.[11] The following probes were used: genomic fragments corresponding to the β, δ, and $\psi\beta$ globin genes; the RIH probe, a 0.5-kb genomic fragment ~4 kb 5′ to the δ globin gene[24]; the RK 29 probe, a 1.2-kb fragment ~18 kb from the 3′ side of the β globin gene[25]; the HPFH-3D and HPFH-3E probes, two genomic fragments from the 3′ endpoint of the Negro HPFH type I deletion[12]; the 7.6 probe, a 0.8-kb fragment ~6 kb 3′ to the deletion endpoint of Negro HPFH type I[12]; a 0.5 *Hind* III unique fragment ~25 kb 5′ to the 3′ endpoint of Negro HPFH type I, obtained from the p3′N10R plasmid (D. Mager, personal communication, 1986) and a 0.75-kb *Hinf I-Eco*RI fragment derived from a plasmid containing a 1.1 kb *Bam* HI–Bgl II genomic fragment, obtained from the 3′ end of an Indian HPFH deletion and mapping at ~30 kb 3′ to the β globin gene[22] (Fig 1).

RESULTS

Italian patients with HPFH have almost normal Hb, MCV, and MCH values (Table 1). Except for patient B I-1, who showed 14% Hb F, all the other subjects had Hb F values ranging between 21 and 30% with a pancellular distribution and $G_\gamma/G_\gamma + A_\gamma$ ratios of 0.31 to 0.39 (mean 0.35). Hb A_2 was slightly decreased in all cases. Globin chain synthesis always gave unbalanced β/α ratios, but almost

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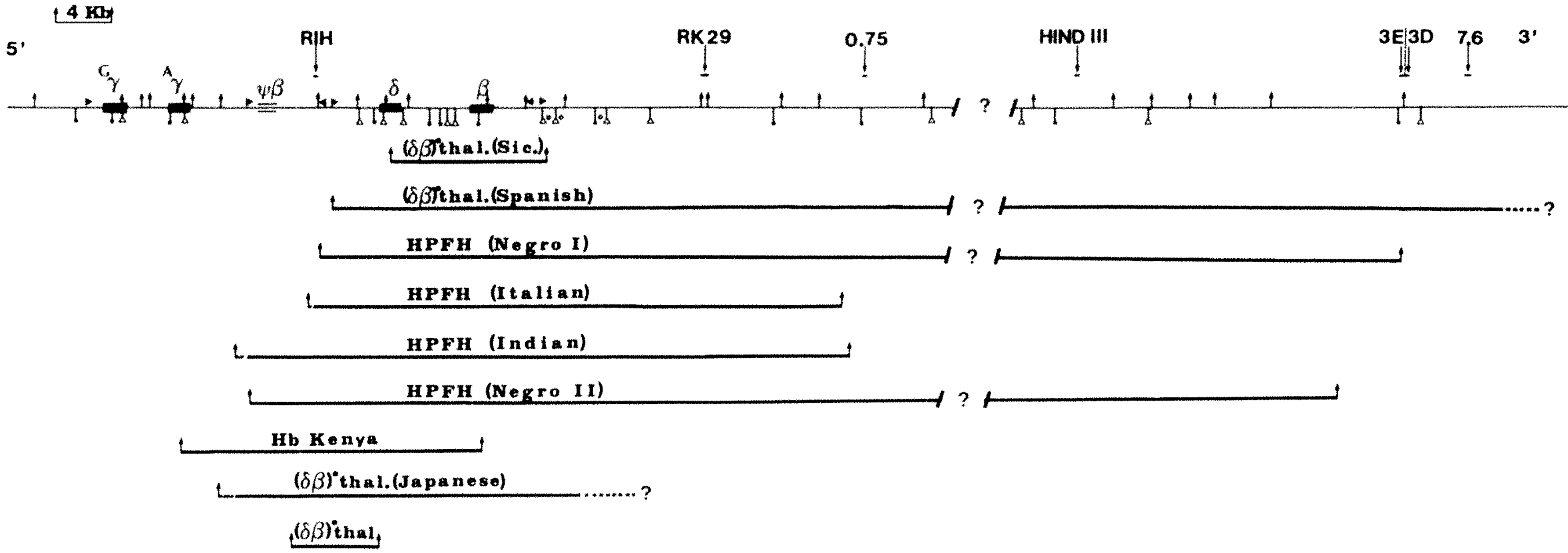


Fig 1. Comparison of the extent of several deletions in the β globin gene cluster causing G_γ/A_γ hereditary persistence of fetal hemoglobin (HPFH) and $\delta\beta$-thalassemias. The restriction map of the region 3′ to the β globin gene cluster was derived from refs. 12 and 19. Probes used in this study are indicated above the DNA map. Short arrows indicate the polarity of the Alu I repeats. The region indicated by a question mark is of unknown length. Extensions of the different deletions are indicated by black bars. Shaded areas indicate uncertainties about the precise location of the breakpoints. ♦, *Bam* HI; ↑, *Eco*RI; △, *Hpa* I restriction sites. The *Hpa* I and *Bam* HI sites marked by asterisks are polymorphic in normal DNA.

Table 1. Hematological Data of Carriers of Italian HPFH

Case	Sex	Age (yr)	Hb (g/dl)	MCV (fl)	MCH (pg)	Hb A_2 (%)	Hb F (%)	β/α (cpm)	$\beta + \gamma/\alpha$ (cpm)	$G_\gamma/g_\gamma + A_\gamma$*
Family A										
I.1	F	30	11.8	81	25	1.8	21	0.63	0.86	0.31
Family B										
I.1	F	29	13.7	79	25	1.8	14	—	—	0.35
II.1	F	5	12.2	76	24	2.0	21	0.60	0.87	0.33
II.2	F	3	12.0	78	25	1.9	22	—	—	0.39
Family C										
I.1	M	52	13.4	74	28	1.9	30	0.59	0.90	—
I.3	F	48	13.0	90	32	1.7	29	—	—	—
II.1	F	19	12.9	91	29	1.8	28	0.64	0.94	0.37
II.2	M	21	16.6	83	30	1.7	27	—	—	—
II.3	F	20	15.4	84	27	1.8	30	—	—	—

*$G_\gamma/G_\gamma + A_\gamma$ are obtained by isoelectric focusing separation.

HPFH, hereditary persistence of fetal hemoglobin.

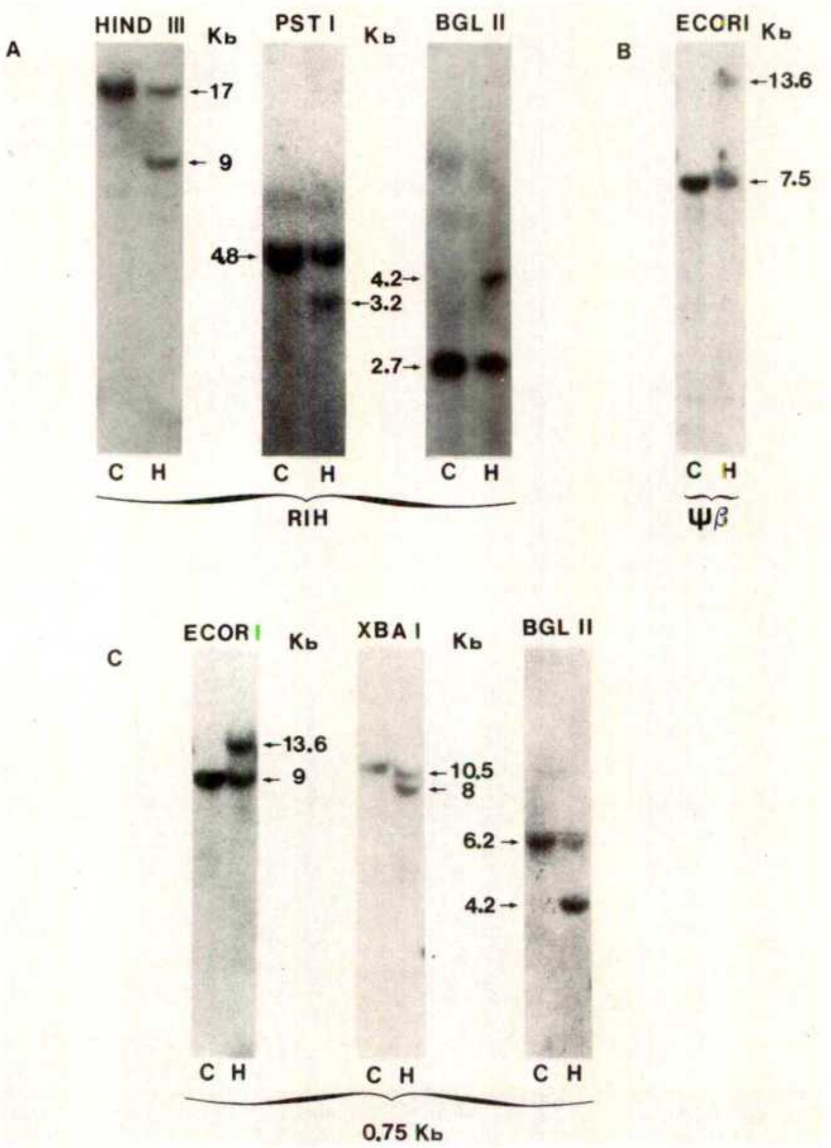


Fig 2. Analysis of the DNA restriction fragments in an Italian hereditary persistence of fetal hemoglobin (HPFH) heterozygous carrier (H) and a normal control (C) with the (A) RIH, (B) $\psi\beta$, and (C) 0.75-kb probes.

Table 2. DNA From Italian HPFH Carriers and Normal Controls Digested With Different Restriction Enzymes and Hybridized With RIH, ψβ and 0.75-kb Probes

Probe	Enzyme	DNA Fragment Size, kb Italian HPFH*	 Normal
RIH	*Pst* I	3.2	4.8
	Bgl II	4.2	2.7
	Hinc II	12.0	7.6†
	Bam HI	14.0	15.0
	Hind III	9.0	17.0
ψβ	*Eco* RI	13.6	7.2
	Hpa I	17.6	14.0
0.75	*Eco* RI	13.6	9.0
	Hpa I	17.6	26.0
	Hinc II	12.0	11.5
	Xba I	8.0	10.5
	Bgl II	4.2	6.2

*Only the size of the abnormal fragments is reported.

†The two *Hinc* II sites 5′ to the Alu region are polymorphic in normal DNA, generating with the RIH probe fragments of different length (1.6, 4.6, and 7.6 kb, respectively).

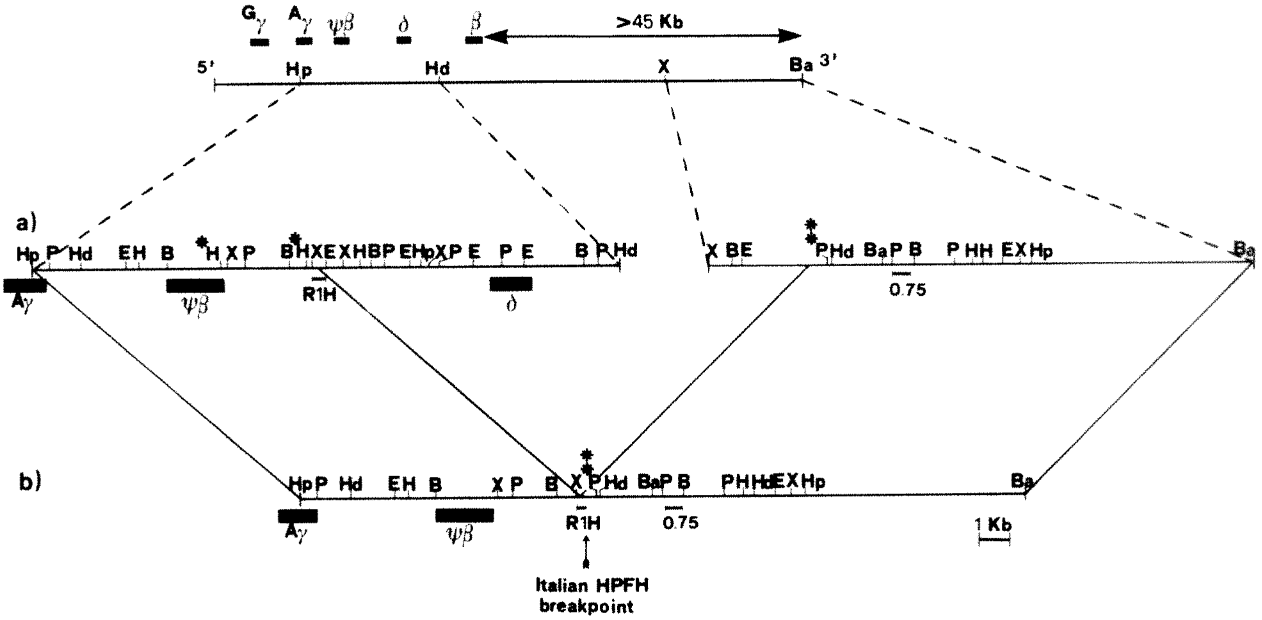
HPFH, hereditary persistence of fetal hemoglobin.

normal $\beta + \gamma/\alpha$ ratios, due to the high level of γ chain production.

Individuals from all three families were studied by Southern blot techniques. Digestion with several restriction enzymes and hybridization to the β and δ probes did not show any abnormal fragments, ruling out the previously described Sicilian $\delta\beta^{\circ}$-thalassemia[5]: on the other hand, the low intensity of the normal bands suggested complete deletion of the $\delta\beta$ globin cluster on the HPFH chromosome (not shown).

Using the RIH probe, digestion with *Pst* I, *Bgl* II, *Hinc* II, *Bam* HI, and *Hind* III (Fig 2A and Table 2), in addition to normal fragments, gave abnormal bands usually of lower intensity as compared with the normal ones. The same abnormal fragments (with the exception of the *Bgl* II digest) could be visualized using a 4.2-kb *Bgl* II ψβ probe, although in this case the intensity of the abnormal band was comparable to that of the normal one (not shown). In addition, the ψβ probe demonstrated an abnormally high-mol-wt *Eco*RI fragment that we were unable to detect clearly with RIH (Fig 2B and Table 2). These data suggest that the *Eco*RI site immediately 3′ to the RIH region may be missing, and that the deletion begins somewhere within the 500-bp RIH sequence, thus explaining the faintness of the abnormal fragments obtained with this probe. As will be shown later, the same abnormal *Eco*RI fragment is detectable with a probe mapping to the 3′ end point of the deletion.

To map the 3′ endpoint of the deletion, six probes located at different distances from the 3′ side of the β globin gene were used (pRK29, 0.5 *Hind* III derived from the p3′Nior, 0.75-kb probe, HPFH 3E, HPFH 3D, and 7.6). The 0.75-kb fragment excised from the plasmid *Bam* HI-Bg II 1.1, mapping immediately 3′ to the deletion endpoint of an Indian HPFH[22] ~30 kb 3′ to the β globin gene, demonstrated abnormal bands in *Eco*RI, *Hpa* I, *Hinc* II, *Xba* I, and *Bgl* II digest of HPFH DNA (Fig 2C and Table 2). Rehybridiza-


Gγ
Aγ
ψβ
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>45 Kb
5′
Hp
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a)
Hp P Hd EH B *H X P B*HXEXHBP EHpXP E P E B P Hd
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Aγ
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R1H
δ
0.75
b)
HpP Hd EH B X P B X **P Hd BaP B PHHdEXHp Ba
Aγ
ψβ
R1H
0.75
1 Kb
Italian HPFH breakpoint

Fig 3. Map of the regions corresponding to the 5′ and 3′ breakpoints of the Italian hereditary persistence of fetal hemoglobin (HPFH) deletion, in normal (a) and in HPFH (b) chromosomes. The position of the restriction sites of the region corresponding to the 0.75-kb probe was derived partially from ref. 22 (*Eco* RI, *Bam* HI, and *Hpa* I sites). Ba, *Bam* HI; Hp, *Hpa* I, Hd, *Hind* III; H, *Hinc* II; B, *Bgl* II; E, *Eco*RI; P, *Pst* I, X, *Xba* I. The *Pst* I abnormal site marked with a double asterisk (detected by the RIH and not visible with the 0.75-kb probe) was precisely mapped by *Bgl* II-*Pst* I and *Xba* I-*Pst* I double digestions (data not shown). The *Hinc* II sites indicated by a single asterisk are polymorphic in normal DNA. The position of the 3′ *Hinc* II site on the HPFH chromosome was obtained by *Bam* HI–*Hinc* II double digestion and hybridization with the 0.75-kb probe; the 5′ *Hinc* II site was deduced on the basis of the length of the *Hinc* II abnormal fragment. Data are consistent with the absence of both *Hinc* II polymorphic sites in the HPFH DNA.

tion of the same filters with the $\psi\beta$ probe identified, as expected, the *Eco*RI, *Hpa* I, and *Hinc* II abnormal fragments with those observed with the 0.75-kb probe. The *Bgl* II abnormal fragment comigrated with the one detected by the RIH probe. However, as already reported for the *Eco*RI digestion, with the RIH probe we were unable to find the abnormal Xba I fragment clearly detectable with the 0.75-kb probe.

Figure 3 shows restriction sites detectable in normal DNA with the 0.75-kb probe, and compares them with the map for the HPFH DNA. Assuming that the 5′ end of the deletion lies somewhere within the 500 bp of the RIH region, the length of the abnormal *Hind* III, *Eco* RI and *Bgl* II abnormal fragments places the 3′ end of the deletion at ~2 kb 5′ to the 0.75-kb probe.

The 5′ endpoint of a deletion found in Spanish $\delta\beta°$-thalassemia is close to the one observed in our HPFH and Negro HPFH type I[9]; it was of interest to investigate if the 3′ endpoint in this condition was in close proximity to that found in these HPFHs. DNA from a homozygous patient with Spanish $\delta\beta°$-thalassemia does not hybridize to probes HPFH 3D and to probe 7.6, although a $\psi\beta$ probe clearly detects the region 5′ to the breakpoint when hybridized to the same filter (data not shown).

A comparison of the restriction enzyme map of Spanish $\delta\beta°$-thalassemia[11] with the map of cloned DNA from the region corresponding to the 3′ breakpoint in Negro HPFH type I indicates that the deletion in Spanish $\delta\beta°$-thalassemia must extend at least 2.0 kb 3′ to the 7.6 probe, as shown by the lack of concordance between *Pst* I and *Bgl* II sites 3′ to the 7.6 in normal DNA and sites 3′ to the breakpoint in Spanish $\delta\beta°$-thalassemia DNA (Fig 4). We cannot exclude however, that the *Hind* III and *Bgl* II sites at the 3′ end of the normal map may correspond to similar sites in Spanish $\delta\beta°$-thalassemia DNA. Unfortunately, a DNA fragment derived from the 3′ site of the breakpoint in Spanish $\delta\beta°$-thalassemia shows the presence of repetitive sequences, which precludes its use in genomic blotting studies.

DISCUSSION

The data reported in this paper are relevant to models proposed to interpret different levels of γ chain synthesis in HPFH and $\delta\beta$-thalassemia on the basis of different deletions. Analysis of the DNA from Italian HPFH carriers reveals that the deletion begins immediately 5′ to the two Alu I repeats upstream of the δ gene, ending ~30 kb 3′ to the β globin gene.

Of the three hypotheses outlined in this report, at least one can now be ruled out by our data; Fig 1, which compares several deletions causing $G_\gamma A_\gamma$ HPFH and $\delta\beta$-thalassemias, clearly indicates that the overall extent of the deletion is not related to the phenotype, as deletions causing $\delta\beta$-thalassemia may be both longer (Spanish $\delta\beta°$-thalassemia, present article) and shorter (Sicilian $\delta\beta°$-thalassemia)[5] than are several HPFHs.

An additional hypothesis suggests that an enhancer sequence (or any other elements capable of modifying gene expression), originally located at some distance 3′ to the β globin gene region, may be brought into the γ globin region by the deletion.[12] Any such sequence existing 3′ to the Negro HPFH type I breakpoint would have the potential to stimulate γ globin gene expression in Negro HPFH type I and II and would be lost in Spanish $\delta\beta°$-thalassemia. It is difficult to conceive, however, that the same element would be responsible for the HPFH phenotype in our case as well as in Indian HPFH, because their 3′ breakpoints are >30 kb away from the region that might contain this element. Nevertheless, the remarkable coincidence of pairs of 3′ endpoints of different HPFH (Indian and Italian, HPFH I and II) may suggest that an additional element capable of modifying gene expression could exist immediately 3′ to the Indian–Italian HPFH endpoint. In conclusion, plural mechanisms rather than a single mechanism may be required to explain all the different phenotypes.

An alternative hypothesis focuses attention on the 5′ endpoint of the deletions. With the present case, five different deletion-type HPFH syndromes are now known (four G_γ/A_γ HPFH plus Hb Kenya), all of which largely or completely remove, in addition to parts of the β globin cluster, an Alu region ~4 kb 5′ to the δ globin gene. Although it is clear that persistent expression of γ globin genes does not require deletion of this region (as in various $\delta\beta$-thalassemias), it has been proposed that the additional loss of these sequences may raise γ globin expression to the high levels observed in HPFH.[9,10] This idea is not contradicted by our new HPFH; however, two recently reported cases[17,18] show that certain deletions including the Alu region do not necessarily increase γ globin expression to very high levels. A Japanese $\delta\beta$-thalassemia was recently reported in which Hb F production was low (5% to 12%) in spite of the deletion of

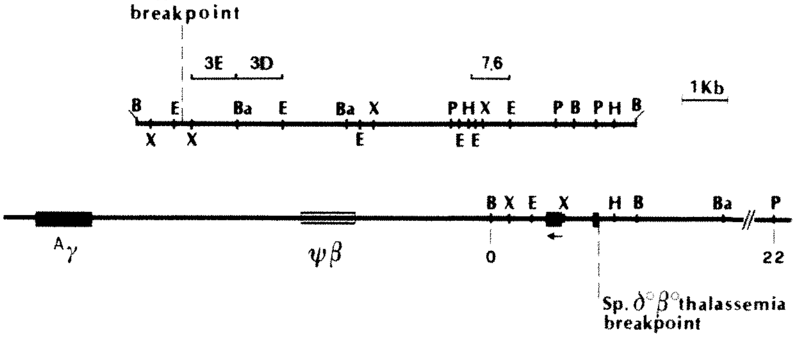

Fig 4. Comparison of the restriction enzyme sites 3′ to the breakpoint in Negro hereditary persistence of fetal hemoglobin (HPFH) type I and Spanish $\delta\beta°$-thalassemia. The map for HPFH was derived from the normal and HPFH DNA clones in ref. 12. The $\delta\beta°$-thalassemia map is taken from refs. 9 and 11. Probes used in the present experiment are indicated. Alu I sequences in Spanish $\delta\beta°$-thalassemia DNA are indicated by a solid square and rectangle (5′ and 3′ Alu repeat), respectively. B, *Bgl* II; X, *Xba* I; E, *Eco*RI; Ba, *Bam* HI; H, *Hind* III; P, *Pst* I.

the Alu sequences.[18] It was remarked, however, that in this case the deletion extended in close proximity to the γ globin gene domain, possibly affecting its expression, particularly that of the A_γ globin gene. In addition, it is now clear that the simple deletion of the Alu region not including the whole $\delta\beta$ globin cluster in a type of $\delta\beta$-thalassemia does not in itself significantly increase the Hb F level, at least in the heterozygotes; a homozygote for this type of $\delta\beta$-thalassemia shows an exceptionally high level of Hb F production, however.[17]

Both the overall lengths of the deletion in Italian and Indian HPFH are very similar, with their 5′ and 3′ ends being roughly in the same regions. A similar situation has been described for the Negro HPFH I and II and on the basis of these and other similar findings, Vanin and colleagues[26] proposed a model for explaining the mechanism of these deletions, suggesting that distant sequences may be brought into proximity during DNA replication, generating a loop that occasionally may be excised. Our findings further support the idea that common mechanisms may operate in generating specific classes of deletions.

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Chromosome 3q21 Abnormalities Associated With Hyperactive Thrombopoiesis in Acute Blastic Transformation of Chronic Myeloid Leukemia

By R. Bernstein, A. Bagg, M. Pinto, D. Lewis, and B. Mendelow

Two patients with acute blastic transformation of chronic myeloid leukemia (CML) associated with strikingly elevated platelet counts showed abnormalities of chromosome 3q in addition to the standard Philadelphia (Ph^1) chromosome translocation. The first patient had an inversion of chromosome 3 (q21q26) cytologically identical to an inversion 3 previously reported in de novo acute megakaryoblastic leukemia, and the second patient showed a translocation between chromosome 3q and the chromosome 9 homologue not involved in the Ph^1 translocation, [t(3;9)(q21;q34)]. Previous studies had incriminated either 3q21 or 3q26 as the locus for a regulatory thrombopoietic gene, but the current study suggests that 3q21 is the relevant site.

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THE SPECIFIC ASSOCIATION of chromosome 3q homologous translocation[1-7] or paracentric inversion of 3q[3,5-8] with acute nonlymphocytic leukemia (ANLL) characterized by abnormal thrombopoiesis suggests that these 3q abnormalities identify a particular subtype of ANLL with features of acute megakaryoblastic leukemia (?M7).[3,8] The French-American-British (FAB) classification of acute leukemia has recently been amended to include an M7 category.[9]

The breakpoints common to these 3q abnormalities were 3q21 and 3q26 (or 3q25). An elevated serum thrombopoietin level in one of the aforementioned patients with acute megakaryoblastic leukemia and inv(3)(q21q26)[8] led to the suggestion that either 3q21 or 3q26 contains the locus of a gene regulating thrombopoiesis.

We here report on two patients with Philadelphia (Ph^1) chromosome–positive chronic myeloid leukemia (CML) in whom acute blastic transformation associated with markedly elevated platelet counts developed. Abnormalities of 3q were again found; inv(3)(q21q26) was noted in one patient and a t(3;9) (q21;q34) in the other. The common breakpoint on chromosome 3 is 3q21.

CASE REPORTS

Patient M.M. A 59-year-old white female presented in Sept 1982 with the clinical and hematologic features of chronic-phase CML. Details of the patient's subsequent course and therapy are presented in Figs 1 and 2. The chronic phase was unremarkable except for a course punctuated by an extremely high platelet count that was difficult to control. In July 1984 a platelet count of 2,950 × 10^9/L necessitated treatment with 5 mCi ^{32}P (Fig 1).

In Feb 1985 she showed acceleration of her disease, and in Aug 1985, 35 months after initial presentation, acute blastic transformation was observed. The platelet count at the time was >1,000 × 10^9/L (Fig 2). Marrow aspirate tests revealed 38% blasts that had an undifferentiated morphology (Fig 3A) and were negative for myeloperoxidase, Sudan black, and periodic acid–Schiff (PAS). They did not express common acute lymphoblastic leukemia antigen (CALLA) or myeloid antigens, as defined by the monoclonal antibodies J5 and MY 906, respectively, and were negative for terminal deoxynucleotidyl transferase (TdT), but 68% of mononuclear cells expressed the Ia antigen. Unfortunately, neither platelet peroxidase nor platelet monoclonal antibodies were used in this study.

An induction course of DOAP was followed by an initial pancytopenia, but the WBC count rose again within a week to 123 × 10^9/L with 30% blasts, and the platelet count reached a level of >2,000 × 10^9/L. A marrow aspirate contained 50% blasts at this stage. A marrow biopsy specimen consisted almost entirely of solid sheets of abnormal megakaryocytes, including numerous micromegakaryocytes (Fig 3B). There was a marked increase in background fibrosis. The patient was not given any further chemotherapy and died of renal failure and septicemia in Sept 1985, 5 weeks after transformation to the acute phase.

Patient D.T. A 32-year-old black manual worker presented at a peripheral hospital in Oct 1982 with a 5-month history of left upper abdominal pain. There was no known occupational or therapeutic exposure to carcinogens. A diagnosis of CML in the chronic phase was made on the basis of his clinical presentation and hematologic findings. He received busulphan therapy for 2 months but was subsequently lost to follow-up for 6 months.

He again presented in June 1983 when he was acutely ill. On examination, he had a lobar pneumonia, numerous subcutaneous nodules (possibly infiltrative), generalized lymphadenopathy, a 10-cm splenomegaly, and a 4-cm hepatomegaly. Blood cultures revealed a staphylococcal septicemia. Peripheral blood indexes were indicative of acute blastic transformation of CML. The hemoglobin concentration was 5.5 g/dL, and the WBC count was 550 × 10^9/L with a differential count of 69% blasts, 3% promyelocytes, 2% myelocytes, 3% metamyelocytes, 15% neutrophils, 5% basophils, 1% eosinophils, 2% lymphocytes, and five normoblasts per 100 white cells. Auer's rods were not observed. The platelet count was 1,028 × 10^9/L. Bone marrow aspiration yielded a dry tap. Bone marrow trephine biopsy results showed a marked, diffuse increase in cellularity, predominantly because of a pronounced proliferation of blasts and megakaryocytes that were clonally distributed; micromegakaryocytes were prominent (Fig 4). Focal residual areas of granulopoiesis with maturation were present. Erythropoiesis was markedly depressed. The patient received packed red cells, antibiotics, and a single dose of chemotherapy consisting of prednisone, hydroxyurea, adriamycin, and vincristine, but died of septicemia five days after admission.

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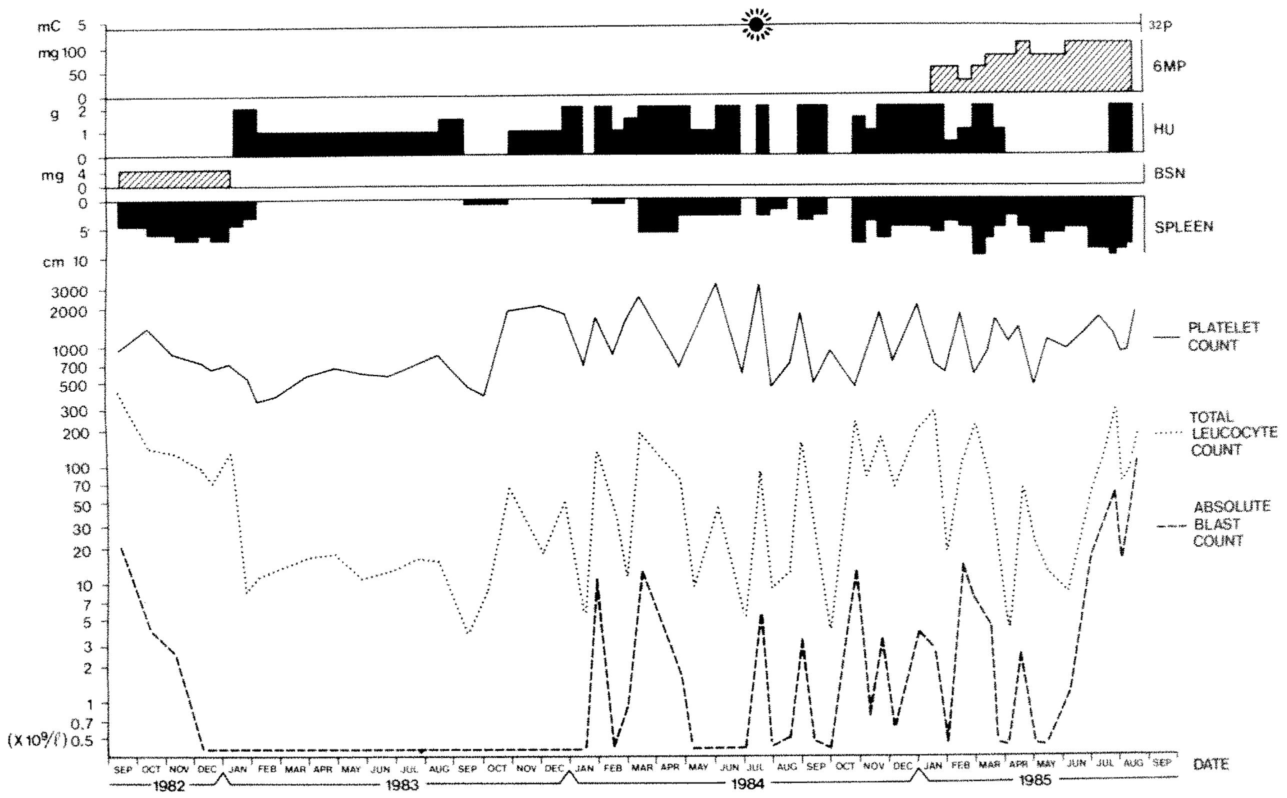

Fig 1. Clinical and hematologic course of patient M.M. during the chronic and accelerated phases. Abbreviations: SPLEEN, splenic size below the left costal margin; BSN, busulphan; HU, hydroxyurea; 6MP, 6-mercaptopurine.

MATERIALS AND METHODS

Metaphases were obtained from the bone marrow aspirate by direct processing and a 24-hour unstimulated culture. Unstimulated and control phytohemagglutinin (PHA)-stimulated 48- and 72-hour cultures were established from peripheral blood. Chromosome analyses were performed on Giemsa- (GTG) and quinacrine (QFQ)-banded metaphases. An abnormal clone was defined as at least two cells with the same structural abnormality or the same extra chromosome and at least three cells with the same missing chromosome. Random loss of chromosomes was not specified in the results.

RESULTS

Patient M.M. Initial chromosome studies performed when the patient first presented in Sept 1982 revealed a Ph^1-positive karyotype of 46,XX,t(9;22)(q34;q11). No other abnormalities were noted, but the chromosomes were short, and their banded morphology was poor. Cytogenetic studies were not repeated until July 1985 when the patient was in the accelerated phase of her disease. At this stage the karyotype appeared to be unchanged, but chromosome preparations were again of poor quality. The final analysis was performed in Sept 1985 when acute blastic transformation had already occurred. All 29 cells analyzed had the Ph^1 translocation, but in addition, 25 of these cells showed an inversion of 3q, karyotype 46,XX,t(9;22),inv(3)(q21q26). (Table 1 and Fig 5). No polyploid metaphases were detected in over 100 cells systematically screened. Metaphases from previous chromosome analyses were retrospectively reviewed, and in two of 13 cells analyzed during the accelerated phase, the inv(3q) was detected.

Patient D.T. Initial chromosome studies at presentation were unsuccessful. Chromosome analysis during the acute phase of his illness, 8 months later, revealed 13 cells with a standard Ph^1 translocation, t(9;22), and they all showed another translocation of chromosome 3q to the chromosome 9 homologue not involved in the Ph^1 translocation, t(3;9)(q21;q34). In seven of these cells clonal evolution was noted, with duplication of the chromosome 9 derivative of the t(3;9), disomy Y (confirmed by the quinacrine fluorescence method), and trisomies of chromosomes 8, 19, and 21 (Table 1 and Fig 6). Three normal cells with a 46,XY karyotype were found among PHA-stimulated metaphases. No polyploid metaphases were detected.

DISCUSSION

The association of megakaryocytic and thrombopoietic abnormalities observed in patient M.M. together with a cytologically identical inv(3)(q21q26) to that found in previously described cases of de novo acute leukemia with abnormal thrombopoiesis,[3,5-8] is most unlikely to be coincidental. This inversion therefore points to a gene situated either at 3q21 or 3q26 that is intimately involved in the regulation of normal thrombopoiesis. Bitter et al[6] suggest that whether the abnormality of 3q is an inv(3q), a t(3q;3q), or an ins(5;3q) the important event in determining abnormal

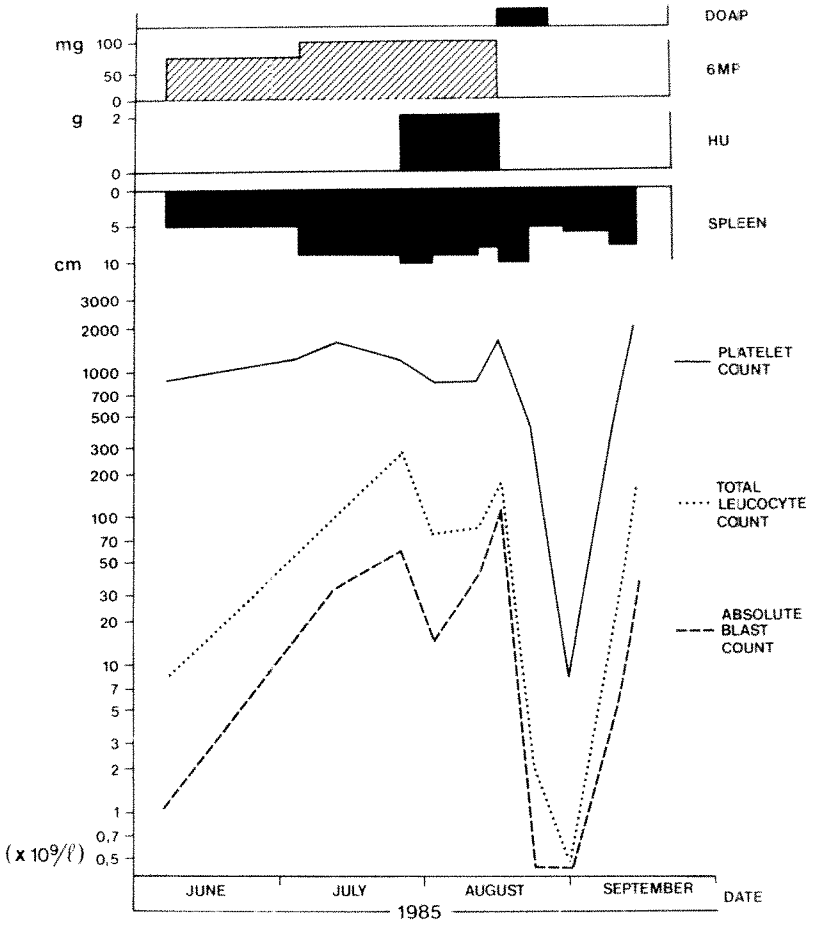

Fig 2. Clinical and hematologic course of patient M.M. during the acute phase. Abbreviation: DOAP, daunorubicin, 50 mg/d in an intravenous injection (IVI) for three days; + vincristine, 2 mg/wk IVI for two doses; + cytosine arabinoside, 200 mg/d IVI for five days; + prednisolone, 100 mg/d, orally (PO) for ten days.

thrombopoiesis is the aberrant juxtaposition of genes on 3q21 and 3q26. The t(3;9) found in patient D.T. affects only the q21 breakpoint on chromosome 3, localizing region 3q21 as the more likely site for the locus.

The translocation detected in patient D.T. is of great interest because the translocated segment 3q21−>3qter was attached to the same 9q34 region cytogenetically as the Ph^1 translocation, but in this instance, affected the 9 homologue not involved in the t(9;22). The recent molecular identification of a new fusion gene on the Ph^1 chromosome by the transposition of the oncogene c-*abl* from 9q34 to the *bcr* of 22q11[10] clearly incriminates both the 9q34 and 22q11 breakpoints in the pathogenesis of CML and affords a rational explanation for a series of cytogenetic observations that had been made previously.[11] In this case, transposition of a thrombopoietic gene from 3q21 to 9q34 could have been an important mechanism in dictating the type of evolution in patient D.T. instead of the usual juxtaposition of 3q21 and 3q26 as suggested by Bitter et al.[6] Furthermore, the duplication of this 9 derivative of t(3;9) may be analogous to the duplication of the Ph^1 chromosome frequently observed in the progression of CML,[12] even when present in a masked form.[13]

A review of karyotypic findings in previously documented cases of CML with suspected or confirmed megakaryoblastic transformation[7,14-30] was informative in only two instances. Carbonell et al[27] reported a patient with Ph^1-positive CML in whom an ins(3;3)(q26;q21q26) was identified during blast crisis; this patient's course was characterized by hyperplasia of morphologically abnormal megakaryocytes and thrombocythemia, but decreased megakaryocytes and thrombocytopenia were observed when blast crisis supervened. Pintado et al[7] described a patient with blastic transformation of Ph^1-positive CML and thrombopoietic abnormalities associated with an inv(3)(q21q26). These reports and our own suggest a possible utility for the cytogenetic identification of megakaryoblastic transformation.

The other cases were not contributory in assessing whether chromosome 3q abnormalities were present or not. All cases, with one exception,[15] were Ph^1-positive, but the only published karyotype was unbanded.[22] Abnormalities such as a paracentric inversion of 3q would not be detected in unbanded or poorly banded chromosomes (for instance, the earlier studies performed on patient M.M.).

On the other hand, a review of previously described chromosome 3q abnormalities in blastic CML were not definitive in establishing an association with abnormal thrombopoiesis. Mecucci et al[5] described three patients with Ph^1-positive CML in blast crises who had a t(3q−;3q+) abnormality and a further four patients in blastic CML with

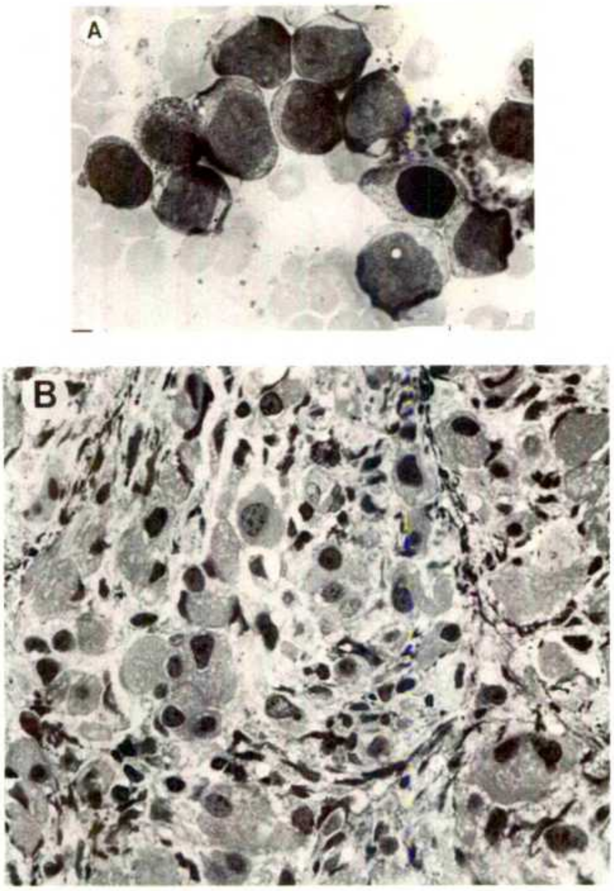

Fig 3. (A) Bone marrow aspirate of patient M.M. at the time of blastic transformation showing undifferentiated blasts and platelet clumping adjacent to one of the dividing blasts (original magnification × 1,250; current magnification × 563). (B) Bone marrow trephine biopsy specimen of patient M.M. 1 week after a course of induction chemotherapy showing sheets of abnormal megakaryocytes, numerous micromegakaryocytes, and marked background fibrosis (original magnification × 500; current magnification × 375).

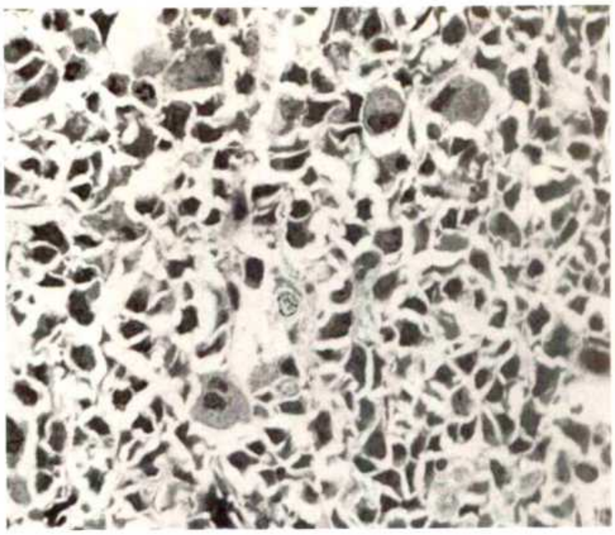

Fig 4. Bone marrow trephine biopsy specimen of patient D.T. at the time of acute blastic transformation showing numerous blasts, many with abundant cytoplasm, interspersed with increased numbers of abnormal megakaryocytes including micromegakaryocytes (original magnification × 500; current magnification × 370).

translocations involving chromosome 3q, but no details concerning thrombopoietic activity are specified. Barlogie et al[31] also recorded a case of Ph^1-positive CML with a t(3;3) during blastic crisis, but here again the characteristics of the transformation are unknown.

In summary, the cytogenetic findings in two cases of CML with transformation, which is very suggestive of a megakaryoblastic acute phase, and previous findings in both de novo acute leukemia or preleukemia with abnormal thrombopoiesis[1-8] and a few cases of blastic CML[7,27] point to a locus on chromosome 3, which is the site of a thrombopoietic regulatory gene. Too few cases have been reported to categorically state that 3q21 is a more likely site than 3q26 and that abnormalities at one or both of these sites are specifi-

Table 1. Chromosome Results

Analysis	Karyotype	Number of Cells Analyzed			
		BM	PB, −PHA	PB, +PHA	Total
Patient M.M.					
9/16/82	46,XX,t(9;22)(q34;q11)	9*			9
7/25/85	46,XX			1	1
	46,XX,t(9;22)		10*		10
	46,XX,t(9;22),inv(3)(q21q26)		2†		2
9/12/85	46,XX,t(9;22)		2	2	4
	46,XX,t(9;22),inv(3)(q21q26)		18	7	25
Patient D.T.					
11/4/82	?				
6/8/83	46,XY			3	3
	46,XY,t(9;22)(q34;q11),t(3;9)(q21;q34)		3	3	6
	51,XYY,t(9;22),t(3;9),+der(9)t(3;9),+8,+19,+21		4	3	7

Abbreviations: BM, bone marrow aspirate culture; PB, peripheral blood cultures.

*Chromosome morphology was too poor to determine retrospectively whether chromosome 3 had an inversion or not.

†Retrospective review of chromosome 3 banding pattern.

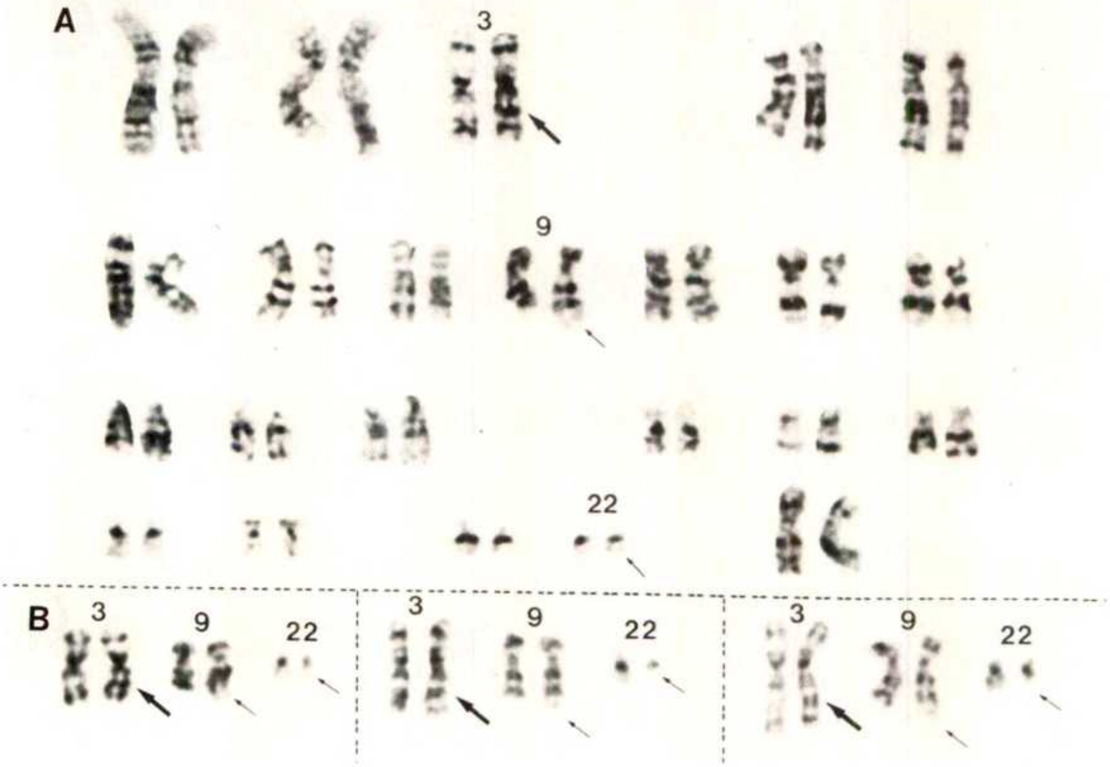

Fig 5. (A) GTG-banded karyotype of patient M.M., 46,XX,t(9;22)(q34;q11),inv(3)(q21q26). (B) Partial GTG-banded karyotypes of three metaphases from patient M.M. showing the inverted 3 (large arrow) and the Ph^1 translocation, t(9;22) (small arrows). (There is a suspicion in the third partial karyotype that both homologues of 3q may be inverted, but the homologue on the left is technically more elongated than the inverted 3q, thus making the diagnosis of an inv(3q) in this homologue uncertain.)

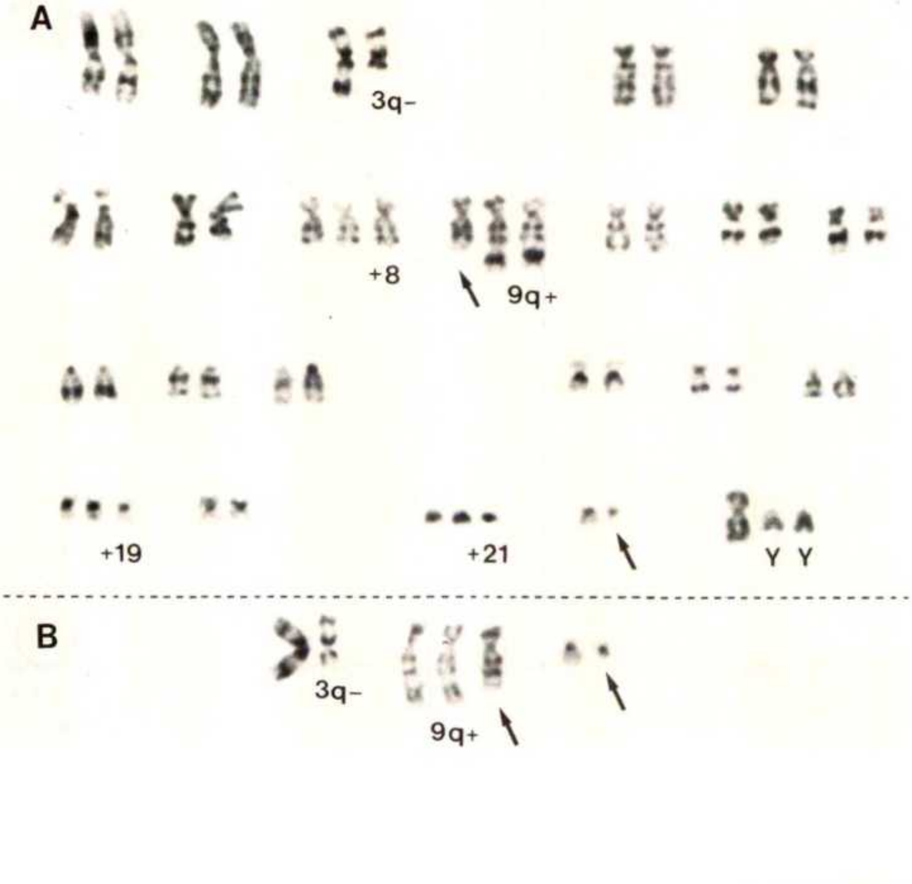

Fig 6. (A) GTG-banded karyotype of patient D.T., 51,XYY,t(9;22)(q34;q11),t(3;9)(q21;q34),der(9),-t(3;9),+8,+19,+21. (B) Partial GTG-banded karyotype of patient D.T. showing the 3q− and the duplicated chromosome 9q+ derivatives of the t(3;9) and the Ph^1 translocation, t(9;22) (arrowed).

cally associated with megakaryoblastic leukemia, but a nonrandom association has been established. This gene could be activated by juxtaposition of 3q21 and 3q26 as suggested by Bitter et al,[6] or alternatively, based on our findings in patient D.T. by the juxtaposition of 3q21 and 9q34. There is a need in future studies to correlate cytogenetic studies with other morphologic, cytochemical, ultrastructural, and immunologic parameters of the blast cell in the acute blastic phase of CML.

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Acute Undifferentiated Leukemia: Implications for Cellular Origin and Clonality Suggested by Analysis of Surface Markers and Immunoglobulin Gene Rearrangement

By Anand Raghavachar, Claus R. Bartram, Arnold Ganser, Gerhard Heil, Enno Kleihauer, and Bernhard Kubanek

Although B cell leukemias and, recently, T cell leukemias can be identified both by surface marker and molecular analysis, there remains a population of acute undifferentiated leukemias (AUL) that cannot be allocated definitively to a single cell lineage. AUL was diagnosed in nine patients according to stringent criteria. We combined both immunologic and molecular approaches to analyze further the ambiguous origin of AUL cells. Southern blot analysis revealed rearranged Ig heavy-chain genes in seven patients and indicated a biclonal or oligoclonal leukemic cell population in three of them, including one case of AUL with translocation (4;11). Analysis of cell surface markers showed expression of at least one early B cell–associated antigen (BA-1, BA-2, B4, UL-38) in six of these seven patients, with coexpression of a myeloid antigen (VIM-2) in three patients. Leukemic cells of two other patients neither exhibited Ig chain gene rearrangements nor expressed B cell-associated antigens. T cell receptor β-chain genes showed germline configuration in all nine cases. Our results demonstrate heterogeneity among AUL patients based on molecular and surface marker analyses and suggest that (*a*) most AUL blast cells are derived from a precursor cell that shares phenotypic and genotypic characteristics of early B cells with certain surface antigens of myeloid cells, (*b*) in some cases of AUL more than one abnormal cell clone or subclone may exist, and (*c*) the cellular origin, at least of some AULs exhibiting t(4;11), may be truly B cell lineage committed.
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PHENOTYPIC ANALYSES of normal and malignant lymphoid cells have considerably improved our current knowledge on lymphocyte differentiation and origin of leukemic cell populations.[1] Four major subtypes of acute lymphoblastic leukemia (ALL) have thus been defined: acute undifferentiated leukemia (AUL), common-type ALL (CALL), B cell ALL (B-ALL), and T cell ALL (T-ALL).[2] Although the cellular origin of AUL remains to be elucidated, the presence of Ig gene rearrangements in all cases of CALLA-positive "non-T, non-B" ALL investigated thus far have given strong support for the hypothesis that the latter cells are neoplastic B cell precursors.[3] Recent reports have shown that Ig gene rearrangements may not be restricted to leukemias of B lineage but may also occur in phenotypically defined acute myeloid leukemias[4] and T-ALL.[5] We therefore combined surface marker and molecular analyses to characterize further the blast cells of nine AUL patients.

MATERIAL AND METHODS

Patients and sample preparations. AUL cell samples were obtained from the Departments of Internal Medicine III and Pediatrics II, University of Ulm. Out of 125 cases of "non-T, non-B" ALL, nine patients showed the following criteria: lack of morphologic signs for myeloid differentiation; negative staining for periodic acid–Schiff (PAS), nonspecific esterase, myeloperoxidase (MPO), and platelet peroxidase (PPO) at the ultrastructural level[6]; lack of reactivity with anti–T cell antibodies (Leu-1, OKT11) and CALLA (J5); and negativity for expression of cytoplasmic Ig. Enriched preparations of blast cells were obtained by Ficoll-Hypaque gradient separation and resulted in $>90\%$ blast cells in all specimens studied.

Immunologic phenotype. The monoclonal antibodies (MoAbs) used for phenotype determination are listed in Table 1. VIM-2 was a generous gift from Dr W. Knapp, Vienna; UL-38 was generated in our own laboratory. All other MoAbs were purchased commercially. MoAbs used in this study were ascites fluids used at saturated binding concentrations; isotype-identical nonreactive ascites served as control. Reactivity with murine MoAbs was determined by standard indirect immunofluorescence staining procedures using fluorescinated rabbit antimouse IgG (FAB')$_2$ or IgM (FAB')$_2$ second antibodies (Cappel Laboratories, West Chester, Pa). For surface immunoglobulin (sIg) detection, cells were stained with affinity-purified goat antihuman Ig antibodies labeled with fluorescein isothiocyanate (FITC) (Cappel). Fluorescence of cells was evaluated using a Zeiss microscope with incident light illumination and equipped for the dual wavelength method. The presence of terminal deoxynucleotidyl transferase (TdT) was examined by indirect immunofluorescence as described by Bollum.[7] Over 200 cells were counted for each examination, and the number of positive cells was expressed as a percentage.

Cytogenetic studies. Karyotype analyses were performed on G-banded preparations as previously described.[8]

DNA analysis. DNAs were extracted from mononuclear cells after Ficoll-Hypaque centrifugation by standard techniques. Fifteen micrograms of DNA was digested with appropriate restriction enzymes (Boehringer Mannheim, Mannheim, FRG), electrophoresed on a 0.7% agarose gel, blotted, and hybridized as described.[9,10] λ-DNAs were included as molecular weight markers. *Bam*HI and *Hind*III codigests were hybridized to a JH probe; *Bam*HI or *Eco*RI digests were hybridized to a $C_T\beta$ probe (both probes obtained from Oncor, Gaithersburg, Md). Moreover, to demonstrate gene rearrangements within $C\mu$ sequences, *Bam*HI digests of cases 8 and 9 were hybridized to a 1,3 kb *Eco*RI probe, which detects a 17-kb μ germline band in *Bam*HI digests (kindly provided by P. Leder). After hybridization the filters were washed under stringent conditions and exposed to XAR-5 film (Kodak, Rochester, NY) using Dupont (Wilmington, Del) Lightning-Plus intensifying screens for 12 to 48 hours at −70 °C. We have not examined the configuration of Ig light-chain genes since rearrangement of these sequences follows expression of CALLA.[3]

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Table 1. MoABs Used for Phenotyping of AUL

MoAb	Source*	Reactivity Pattern
BA-1	H	Pan B, granulocytes
BA-2	H	Lymphoid/myeloid progenitor cells
B4	C	Pan B, follows HLA-DR (Ia)
B1	C	Pan B, follows CALLA
UL-38	—	Pan B, follows HLA-DR (Ia)
J5	C	CALLA (CD 10)
OKIa	O	HLA-DR (Ia)
Leu-1	BD	Pan T, sIg-positive, CLL
WT1	BD	Pan T, prothymocytes
OKT11	O	Pan T, E rosette receptor
VIM-2	—	Myelomonocytic cells
MY-7	C	Myelomonocytic cells

Abbreviation: CLL, chronic lymphocytic leukemia.

*Commercially available sources: H, Hybritech Inc, San Diego; C, Coulter Immunology, Hialeah, Fl; O, Ortho Pharmaceutical Co; BD, Becton Dickinson, Mountain View, Calif.

RESULTS

Morphologic, cytochemical, and immunologic studies. Leukemic cells of all nine patients showed no morphologic features of myelopoietic lineage by panoptic staining. Cytochemically, blast cells proved to be negative for PAS, MPO, and PPO; the latter result was confirmed by ultramicroscopic analysis. The cells did not form E rosettes and were negative when tested for Ig and cytoplasmic Ig and thus were not mature T or B cells. Table 2 shows a summary of leukemic cell phenotypes. According to our criteria for AUL, leukemic cells lacked expression of CALLA, B1, Leu-1, and OKT11. The main marker profile, found in seven patients, was BA-2+, HLA-DR+, TdT+, in six cases in combination with expression of very early B cell antigens defined by MoAbs B4 and UL-38, implying B cell origin of these leukemic populations. Reactivity for another early B cell–associated antigen, BA-1, was less frequent. Among the B marker–positive group there were three cases staining brightly positive for the myeloid antigen VIM-2 but negative for MY-7. Two patients displayed a HLA-DR+, WT-1+ phenotype, and in these cases no B cell–associated antigens were unequivocally demonstrated. In addition, one of the latter patients (no. 8) strongly coexpressed MY-7 antigen. The other (case 9) bore HLA-DR, BA-2, and TdT in combination with an early marker for T cells (WT-1) and myeloid cells (MY-7).

Ig heavy-chain and T cell receptor β-chain gene analysis. Ig heavy-chain sequences are rearranged in seven patients (Fig 1A, lanes 1 to 7). Although one allele is involved in case 1, patients 2, 3, and 4 show an involvement of both heavy-chain gene alleles: one allele is deleted, the other rearranged. Faint hybridization signals at 5.6 kb in these cases represent in all likelihood contaminations of samples with nonleukemic cell populations. We tend to interpret the hybridization pattern observed in patient 5 as an indication for biclonality, one allele being rearranged in each clone. The alternative possibility that generation of a novel *Bam*HI and *Hind*III site on one allele creates two rearranged fragments was virtually ruled out by *Eco*RI single digests likewise showing two rearranged fragments (Fig 1B); the latter data also argue against a restriction enzyme polymorphism. Three rearranged fragments observed in patient 6 suggest even stronger biclonality of the respective leukemic population. This view was confirmed by an *Eco*RI single digest likewise exhibiting three additional fragments (Fig 1B). Five distinct rearranged fragments are detected in patient 7, indicating oligoclonality. The latter result is neither explained by DNA overdigestion or partial digestion, for partial digests of this sample revealed the same autoradiographic bands, nor by partial degradation of DNA, for in case of partial digest, bands of longer size than the germline fragment should appear. Moreover, both possibilities were virtually ruled out by hybridization of the same filter to $C_T\beta$ sequences (Fig 2B, lane 7) exhibiting only germline fragments. Various rearranged fragments are also visible in an *Eco*RI digest of leukemic cells in this case (Fig 1B). For the group of patients showing more than two rearranged fragments, chromosomal studies could be performed subsequent to molecular analyses. In patients 5 and 6 no chromosomal abnormality was detectable. Notably, blast cells of patient 7 were characterized by translocation t(4;11). Twenty metaphases were ana-

Table 2. Results of Immunologic and Molecular Biologic Analyses in AUL Blast Cells

		Cell Surface Markers*												DNA Analyses†	
Patient	TdT	BA-1	BA-2	B4	B1	J5	UL-38	OKIa	Leu-1	WT-1	OKT11	VIM-2	MY-7	Ig heavy chain	TCR
1	90+	5++	95++	92+	—	—	90+	90++	—	2+	—	74++	—	R	G
2	85+	98++	98++	95+	—	—	98++	96++	96++	—	—	—	—	R	G
3	70++	80++	50+	65+	—	—	80++	90++	—	NT	9+	60++	2+	R	G
4	30+	—	75+	50+	—	—	33++	92++	—	NT	—	34++	—	R	G
5	—	—	—	—	—	—	—	95++	—	65++	—	—	—	R	G
6	98+	90++	98+	70+	—	—	98++	52++	—	—	—	2++	NT	R	G
7	50+	41++	46++	48++	—	—	50+	55++	—	NT	—	—	—	R	G
8	—	—	—	—	—	—	—	99+	—	90++	—	2++	62++	G	G
9	95++	—	90++	—	—	—	—	90++	—	98++	—	5+	56+	G	G

TCR, T cell receptor β chain.

*Percentage of positive cells as determined by indirect immunofluorescence analysis: +, weak fluorescence; ++, strong fluorescence; —, negative; NT, not tested.

†Gene configurations are categorized as follows: G, germline; R, rearranged.

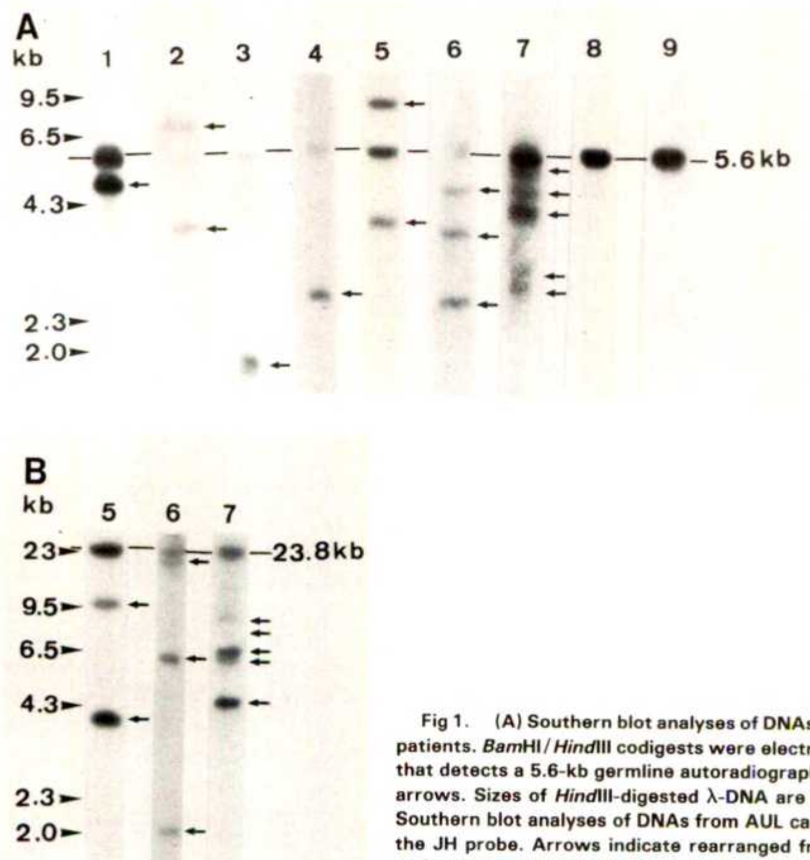

Fig 1. (A) Southern blot analyses of DNAs (15 μg) obtained from leukemic cells of nine AUL patients. *Bam*HI/*Hin*dIII codigests were electrophoresed, blotted, and hybridized to a JH probe that detects a 5.6-kb germline autoradiographic band. Rearranged fragments are indicated by arrows. Sizes of *Hin*dIII-digested λ-DNA are included as the molecular weight standard. (B) Southern blot analyses of DNAs from AUL cases 5, 6, and 7. *Eco*RI digests were hybridized to the JH probe. Arrows indicate rearranged fragments. *Hin*dIII-digested λ-DNA served as the molecular weight standard.

lyzed, and all showed the karyotype 46,XY,t(4;11). Two patients exhibited no rearrangement within the Ig heavy-chain sequences covered by the JH probe (Fig 1A, lanes 8 and 9). In addition, hybridization of these two patient DNAs to a Cμ probe failed to help in establishing the cell lineage of these two cases (Fig 3). Hybridization of the Tβ probe to *Eco*RI or *Bam*HI digests of all nine leukemic cell DNAs showed a germline configuration of T cell receptor β-chain sequences (Fig 2).

DISCUSSION

In contrast to accumulating data supporting the view that CALLA+ leukemic populations are neoplastic B cell precursors, the cellular origin of AUL is still a matter of controversy.[2] Comparison of cell surface marker phenotypes and Ig heavy-chain gene analysis reported here support the recent notion[12] that in the presence of early B cell markers AUL cells show rearranged Ig genes. Moreover, the detection of Ig heavy-chain gene rearrangement in AUL cells with HLA-DR+, B marker− phenotype (case 5) is in agreement with the view that Ig gene rearrangements precede expression of B cell–associated surface antigens. Biphenotypic expression of myeloid and lymphoid immunologic markers (cases 1, 3, and 4) may indicate that the leukemic transformation event in these cases took place in an immature stem cell capable of differentiation into both lineages.[13] Alternatively, respective leukemic cells may be highly atypic and coexpress markers of various differentiation lineages.[14] Application of combined

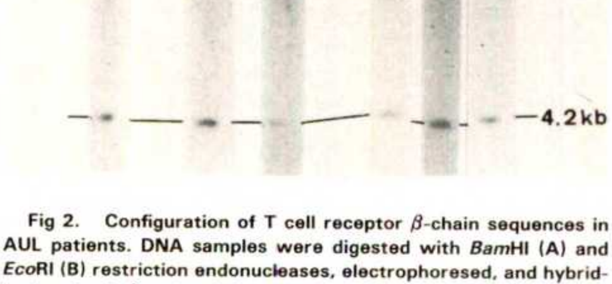

Fig 2. Configuration of T cell receptor β-chain sequences in AUL patients. DNA samples were digested with *Bam*HI (A) and *Eco*RI (B) restriction endonucleases, electrophoresed, and hybridized to the $C_T\beta$ probe. (A) Patients 1 to 9 show a germline band of 23 kb. (B) Similar results were obtained after *Eco*RI digestion: 12-kb $C_T\beta_1$ and 4.2-kb $C_T\beta_2$ germline fragments are detected in samples available from patients 1, 3, 5, 7, 8, and 9. For analysis of the *Eco*RI digest from patient 7, the filter already used for Ig analysis (Fig 1B) was rehybridized to $C_T\beta$ sequences.

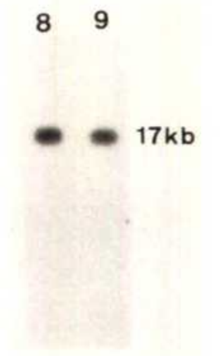

Fig 3. $C\mu$-related sequences of DNAs of patients 8 and 9. *Bam*HI-digested genomic DNA was hybridized to a $C\mu$ probe that detects a 17 kb germline band in *Bam*HI digests.[11]

molecular and immunologic approaches thus suggests a B cell precursor origin in the majority of AUL cases; however, the cellular origin of cases 8 and 9 continues to be uncertain. Neither immunologic nor molecular investigations give sufficient data to allow an unequivocal allocation to alternative lineages. Unusual surface marker profiles in both cases may at best indicate pluripotent stem cell origin and therefore may be truly undifferentiated.

Surprisingly, AUL cases 5, 6, and 7 appear to contain biclonal or oligoclonal cell populations as indicated by Ig gene analysis. This contrasts with the monoclonal pattern observed in all "non-T, non-B" ALLs investigated thus far.[3,12,14] Since patients 5 and 6 exhibited a normal karyotype, trisomy of chromosome 14 can be ruled out as a possible explanation for the hybridization pattern observed in both patients. Furthermore, this seems to be rather unlikely, for AUL is characterized by other specific chromosomal abnormalities.[15] The finding of t(4;11) in all analyzed leukemic metaphases of patient 7 may be of interest. Although Southern blot analysis suggests the presence of an oligoclonal disease in this case, in connection with the karyotype one has to assume that a single B cell precursor underwent transformation. During evolution of this original leukemic clone, additional subclones characterized by specific Ig gene rearrangements but all exhibiting (4;11) may have occurred. The alternative explanation that independent transformation events occurred in unrelated cells carrying different immunoglobulin heavy-chain gene rearrangements appears to be highly unlikely in the context of the chromosomal abnormality of this case. Recent reports have suggested that the cell of origin of the acute leukemia cytogenetically defined by translocation 4;11 may be a common progenitor for B and myelomonocytic cells.[16-18] In this respect, our results differ from those of other groups since case 7 exhibits strong expression of the B cell restricted antigen B4 but lack of myeloid antigens. Taken together, these results suggest that this t(4;11) acute leukemia is a disease of truly B cell–committed lymphoid progenitor cells.

Moreover, AUL as defined may be a different subclass of acute leukemia since a significant adverse influence of the immunologic subtype AUL for the length of complete remission could be shown in a multicenter trial for ALL and AUL with uniform therapy[19] and, in particular, acute leukemia patients with translocation 4;11 appear to have a very poor prognosis.[20] Considering oligoclonal disease in some of these patients, a specific marker of clonality, like detection of Ig gene rearrangement,[21] can be used to monitor the effect of anticancer chemotherapy on specific clones. Moreover, considering another therapeutic approach to tumor therapy involving in vitro purging of bone marrow with MoAbs, molecular analysis may ensure that a MoAb actually reacts with antigens of all leukemic cell clones. Detailed characterization of AUL blasts using both immunologic and molecular approaches thus seems to be essential not only for subclassification of respective leukemic populations to a specific maturation lineage but also for monitoring risk groups in therapeutic studies.

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Lymphoepithelioid Lymphoma (Lennert's Lymphoma) Is a Monoclonal Proliferation of Helper/Inducer T Cells

By A.C. Feller, G.H. Griesser, T.W. Mak, and K. Lennert

Lymphoepithelioid lymphoma (Lennert's lymphoma) was first described as a special variant of Hodgkin's disease. This lesion is characterized by a high percentage of epithelioid and T cells and rarely contains the classical Hodgkin's/Reed-Sternberg cells. Cytogenetic abnormalities indicate that Lennert's lymphoma is of T cell origin. In the present study, immunohistochemical investigation of four cases of Lennert's lymphoma revealed two major cell populations of T cells that predominantly express the helper-inducer phenotype and Ki-M6- and Ki-M8-positive macrophages and epithelioid cells. Double-staining experiments for the detection of cell surface antigens and the proliferation-associated antigen Ki67 showed that only the CD4-positive cells (helper-inducer T cells) were proliferating. Examination of the DNA of these Lennert's lymphoma samples also indicated that monoclonal rearrangement of the T cell receptor β-chain genes has occurred, whereas the immunoglobulin heavy- and κ-chain genes remained in germline configuration. Our results strongly suggest that Lennert's lymphoma is a CD4-positive T cell lymphoma.

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ALTHOUGH THE MAJORITY of malignant lymphomas can be classified as being derived from either the T or B cell lineage, the lineage of certain lesions such as Hodgkin's disease, lymphoepithelioid lymphoma (Lennert's lymphoma), and lymphogranulomatosis X/angioimmunoblastic lymphadenopathy is uncertain. Lymphoepithelioid lymphoma (LEL) was first described by Lennert[1] and later by Lennert and Mestdagh[2] as a special variant of Hodgkin's disease characterized by a high percentage of epithelioid cells and rarely containing the Reed-Sternberg cells characteristic of classical Hodgkin's disease. This disease was called Lennert's lymphoma by Dorfman and Warnke[3] and Lukes and Tindle,[4] and as a result of findings that pointed to a T cell origin for the diseases, the term was changed from lymphoepithelioid cellular lymphogranulomatosis to lymphoepithelioid cell lymphoma. Although cytogenetic studies show chromosomal abnormalities that are in accordance with the designation of a T cell–derived malignant lymphoma and immunohistochemical investigations show a high percentage of T cells[5–7] in this lymphoma, it is not known whether they represent the clonal population responsible for neoplastic proliferation. In this study, we report the results of immunohistochemical analysis of LEL, using double staining with conjugated monoclonal antibodies in an effort to identify the cell surface phenotype of the proliferating neoplastic cell. We have also characterized T cell receptor β-chain and immunoglobulin heavy-chain gene rearrangements in these cells to substantiate the immunohistochemical findings. Our results strongly support the hypothesis that LEL is a T cell lymphoma, probably of the T helper/inducer class.

MATERIALS AND METHODS

Five lymph nodes from four patients with LEL were examined by conventional light microscopy in hematoxilin and eosin (H&E) and Giemsa-stained paraffin sections and classified according to the criteria described by Lennert et al.[8,9] A second biopsy specimen (no. 4b) from one patient was taken 2 years after the onset of the disease. One third of each biopsy sample was used for light microscopy, one third for immunohistochemistry, and one third for DNA hybridization.

Immunohistochemistry. Fresh tissue specimens were snap-frozen in liquid nitrogen and stored at −90 °C. From these samples 7-μm frozen sections were prepared and fixed for ten minutes in acetone. Then, immunohistochemical staining was performed as described by Stein et al.[10] Three nonlymphocytic monoclonal antibodies, KiM1,[11] KiM6,[12] and KiM8[13] were used. Using double-labeling techniques, we stained for both cell surface antigens and Ki67, a nuclear antigen expressed only in proliferating cells.[14,15] Immunoperoxidase staining was performed first to visualize the nuclear antigen (Ki67), and after developing with diaminobenzidine, a second monoclonal antibody was applied to the sections and developed using alkaline phosphatase. Other monoclonal antibodies used in this study are given in Table 1.

Gene rearrangement. Single-cell suspensions were prepared from the snap-frozen lymph node biopsy specimens, and DNA was extracted as described by Minden et al.[16] Southern blot analysis was performed using germline DNA from cultured skin fibroblasts of normal volunteers as a control and DNA from lymphoma cells after overnight digestion with ten units of restriction enzyme (*Eco*RI, *Bam*HI, or *Hind*III) per microgram of DNA. Digested DNA was electrophoresed through 0.6% to 0.8% agarose and transferred to nitrocellulose filters as described by Southern.[17] Filter-bound DNA fragments were then hybridized to nick-translated ^{32}P-labeled gene probes and visualized on autoradiographs.[16] Two probes were used in this study: the constant region of the T cell antigen receptor β-chain characterized by Yoshikai et al[18] and the joining region of the immunoglobulin heavy-chain gene, a gift from P. Leder.[19]

RESULTS

Four biopsy specimens taken at the onset of the patients' disease, which present identical histologic pictures characteristic of LEL, are described in Fig 1. The biopsy sample from patient 4 (4b) showed a low number of epithelioid cells and a high percentage of large, basophilic cells, which were determined by light microscopy to be atypical T cells (Fig 2).

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Table 1. Monoclonal Antibodies Used in This Study With Their Specificity and Source of Reference

Monoclonal Antibody	Cluster	Specificity	Source/Reference
Leu-4	CD3	All T cells	BD
Lyt-3	CD2	Sheep erythrocyte receptor	New England Nuclear, Boston
Leu-1	CD5	T cells, some B cells	BD
Leu-3a	CD4	Helper/inducer T cells	BD
Leu-2a	CD8	Suppressor/cytotoxic T cells	BD
To15	CD22	All B cells	Stein et al[10]
Ki67		Proliferating cells	Gerdes et al[15]

Abbreviation: BD, Becton Dickinson.

Immunohistochemistry. Immunohistochemically investigated biopsy specimens 1 to 4a were phenotypically identical (Table 2). More than 90% of the T cells expressed the T cell antigens CD3, CD5, and CD2, and the majority of lymphoid cells (>80%) expressed CD4, whereas three of the four samples were composed of less than 10% CD8-positive cells (case 3 contained 20% CD8-positive lymphocytes). All samples contained a high percentage of Ki-M1+, Ki-M6+, and Ki-M8+ epithelioid cells. B cells (CD22+), which were diffusely distributed or found only in small clusters, made up about 5% of all lymphoid cells. The number of proliferating cells (Ki67+) ranged from 10% to 30% in the early biopsy specimens (1 to 4a), but in the last biopsy sample (4b) demonstrated an elevated rate of proliferation that was as high as 60%. Lymphoid cells that were still expressing CD3 and CD2 did not express the CD5 or CD4 antigen of helper/inducer T cells. Double-labeling experiments with Ki67, which labels proliferating cells, and monoclonal antibodies recognizing cell surface antigens clearly showed that proliferating cells expressed T cell antigens CD3, CD5, and CD2 (Fig 3A). As shown in Table 3, less than 3% of CD8-positive lymphocytes in each biopsy sample were positive for Ki67 (Fig 3B), whereas a high proportion of lymphoid cells (30% to 70%) expressed Ki67 and the CD4 antigen simultaneously. Epithelioid cells Ki-M1+, Ki-M6+, or Ki-M8+ were Ki67-negative. The same B cells reacted with anti-IgM or the pan–B cell antibody.

Determination of somatic rearrangement. DNA from these biopsy specimens was examined for rearrangement of the T cell receptor β-chain (TcR_β) and immunoglobulin heavy-chain (IgH) genes. DNA samples were digested with each of the restriction enzymes *Eco*RI, *Hind*III, and *Bam*HI and analyzed by Southern gel analysis. The TcR_β genes had undergone rearrangement in all five DNA samples. Following digestion with *Eco*RI, rearranged bands were detected in samples 2 (patient 1), 3 (patient 2), 4b (patient 4), and 5 (patient 3) at 8.2 kb, 9.4 kb, 10.3 kb, and 8.0 kb, respectively (Fig 4). Except for sample 4 (patient 4), the germline bands at 10.5 kb and 3.7 kb were detected either as a result of the presence of nonlymphoid cells in the lymph node biopsy specimen or of rearrangement of only one allele in the clonal cell population.

In one case, two biopsy specimens were obtained from one patient. Sample 1 (patient 4, biopsy a) contained the DNA from the first biopsy specimen; sample 4 (patient 4, biopsy b) represented DNA from the second lymphoma biopsy 2 years later. The rearrangement pattern in these two samples was different. No rearrangement could be seen in sample 1 following DNA digestion with *Eco*RI, whereas in sample 4, a deletion of the 10.5-kb germline band and a faint rearranged band at 10.3 kb were seen, indicating that, in addition to the

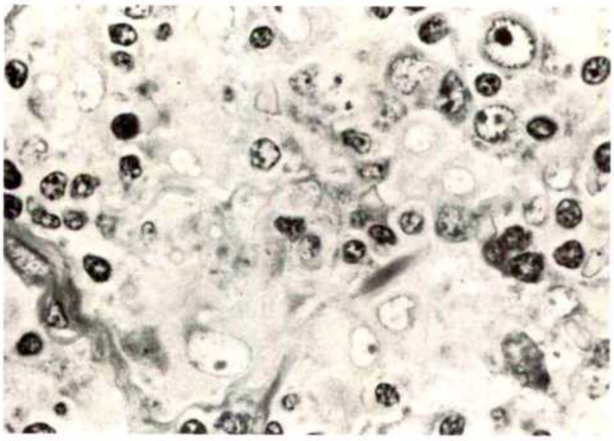

Fig 1. Lymph node biopsy specimen from a patient with Lennert's Lymphoma (patient 4, specimen a). Small lymphoid cells show slight nuclear irregularity. In the lower left is a large cell resembling a Hodgkin's cell. Some epithelioid cells have light nuclei (6-µm paraffin section, Giemsa staining; original magnification × 1024; current magnification × 686).

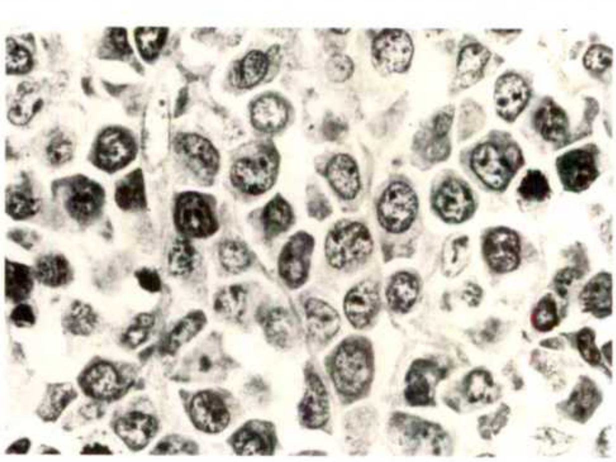

Fig 2. Second biopsy sample from patient 4 (4b). The lymphoma consists of large blastic cells with prominent, slightly basophilic cytoplasm. Epithelioid cells are missing (6-µm paraffin section, Giemsa staining; original magnification × 1024; current magnification × 686).

Table 2. Immunophenotypic Characteristics of Infiltrating Cells in Lymph Nodes With Lennert's Lymphoma

Specimen	CD19 To15	CD3 Leu-4	CD2 Lyt-3	CD5 Leu-1	CD4 Leu-3a	CD8 Leu-2a	Ki67	CD4/CD8 Ratio
1	5	>90	>90	>90	85	5	10	>10:1
2	5	>90	>90	>90	80	3	20	>10:1
3	5	>90	>90	>90	80	20	30	4:1
4a	5	>90	>90	>90	80	5	15	>10:1
4b	5	>90	>90	10	5	2	60	—

Specimens 4a and 4b are from two sequential biopsies performed on the same patient in a 2-year interval. Counts are given in percentages.

Table 3. Percentage of Ki67-Positive Cells That Simultaneously Express a Cell Surface Antigen

	Ki67+ CD22	Ki67+ CD3	Ki67+ CD4	Ki67+ CD8	Ki67+ KiM6	Ki67+ KiM8
1	<3	50	40	<3	<3	<3
2	<3	50	30	<3	<3	<3
3	<3	60	50	<3	<3	<3
4	<3	80	70	<3	<3	<3
4b	<3	70	<3	<3	<3	<3

original rearrangement on one of the alleles, a deletion of $C_{\beta 1}$ took place in the other (Fig 4).[20] This was supported by the finding of $C_{\beta 2}$ rearrangement in sample 4 as well as in sample 1 when *Hind*III was used as the restriction enzyme. Figure 5 shows rearranged bands for sample 1 (patient 4, biopsy a) at 6.9 kb (lane 1) and for sample 4 (patient 4, biopsy b) at 6.5 kb and 4.5 kb (lane 2). No immunoglobulin heavy-chain gene rearrangement could be observed in any of the samples (data not shown).

DISCUSSION

Immunohistochemistry has provided a number of tools for the assignment of cells to the B or T cell lineage. With most malignant non-Hodgkin's lymphomas, if one has a clonal expansion of a monomorphous lymph node infiltrate, these

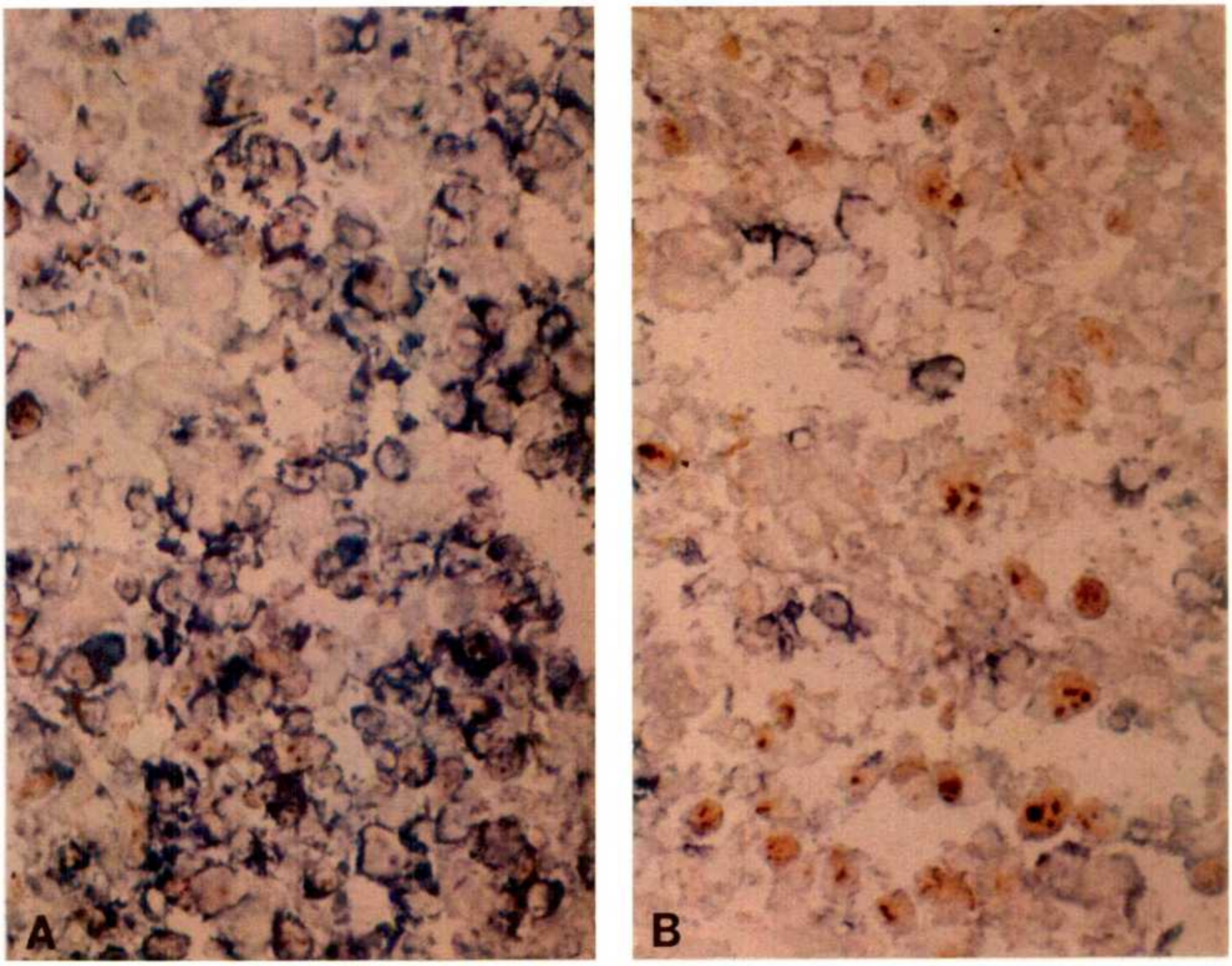

Fig 3. Immunohistochemical double labeling of a lymph node infiltrated by lymphoepithelioid lymphoma. (A) T cells are stained by a CD3 antibody (blue surface staining). Ki67 cells (proliferating cells) show a brown nuclear staining. The majority of Ki67+ cells are additionally positive for CD3 antibody. (B) T suppressor/cytotoxic cells are stained by CD5 antibody (blue surface staining). Ki67+ shows a brown nuclear staining. Not a single cell shows double labeling for both antigens (7-μm cryostat sections, double-staining technique with immunoperoxidase reaction (nuclear antigen Ki67) and immunoalkaline phosphatase reaction for cell surface antigens, no nuclear counterstaining; original magnification × 960).

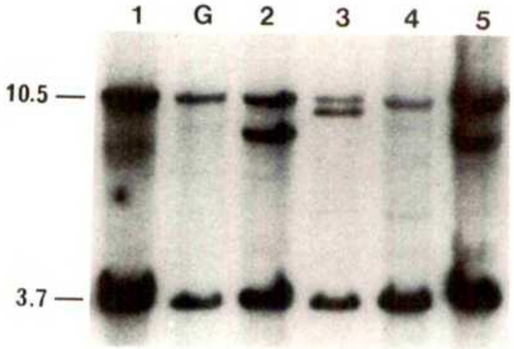

Fig 4. Southern analyses were performed on DNA from lymphoma cells from patients with lymphoepitheloid lymphoma. DNA was restricted with one of the enzymes *Eco*RI, *Bam*HI, or *Hind*III. Filters were hybridized to nick-translated probes of the constant region of the TcR_β and the joining region of the IgH. DNA prepared from cultured skin fibroblasts of healthy volunteers was used as germline control (G). DNA from tissue samples was digested with *Eco*RI and hybridized with the TcR_β probe. Lane 1 (specimen 4a) and lane 4 (specimen 4b) contain samples derived from subsequent lymphoma biopsies from the same patient. Lane 2 is from patient 1; lane 3, patient 2; and lane 5, patient 3. Numbers at the left side denote sizes of the germline fragments in kilobases.

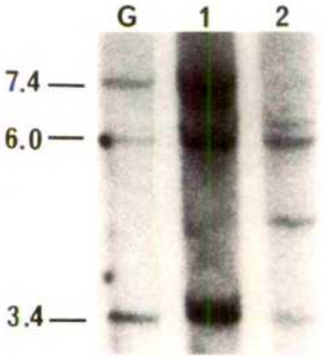

Fig 5. DNA samples from one patient with Lennert's lymphoma digested with *Hind*III and probed with TcR_β. Lane 1 represents the first lymph node biopsy sample (4a) and lane 2 the second one 2 years later (4b).

tools can be used to characterize and phenotype the neoplastic cell clone. In malignant lymphomas such as Hodgkin's disease, which are composed of a mixture of different cell types, it is difficult or even impossible to recognize selectively the neoplastic cell clone and to determine from which lineage it was derived. One such disease is the special variant of Hodgkin's disease lymphoma described by Lennert[1] and later by Lennert and Mestdagh[2] as epithelioid cellular lymphogranulomatosis, later distinguished from Hodgkin's disease and designated lymphoepithelioid cell lymphoma.[9] Recent results indicate that in addition to the high percentage of epithelioid cells there are large numbers of T cells present in this disease[6,7]; the true nature of this disease is still unclear, however, because it remains to be seen whether the T cells compose a clonal neoplastic cell population or whether they only are cells that interact with the neoplastic cells.[21-24]

Histologic and immunohistochemical results show two characteristic cell types in LEL:T cells with the helper-inducer phenotype and epithelioid cells expressing histiocyte antigens. The progression of LEL in patient 4 is remarkable. The first biopsy specimen showed features of LEL, histologically and phenotypically, and the second biopsy specimen, taken 2 years after the onset of the disease, showed features of a high-grade malignant peripheral T cell lymphoma, indicating a shift from a low-grade malignant lymphoma (LEL) to a high-grade malignant lymphoma, a phenomenon known from B cell malignancies. Using a monoclonal antibody that recognized an antigen expressed in all proliferating cells from the late G1 phase to the S phase (Ki67),[15] we tried to define the dominant cell type of the proliferating cells. Double-labeling experiments show that mainly the CD4-positive helper/inducer T cells were proliferating because they were strongly Ki67-positive and the CD8+ lymphocytes and epithelioid cells were Ki67-negative. If, as in other malignancies, the proliferating cells represent the neoplastic cell clone, these results leave little doubt as to the T cell nature of Lennert's lymphoma and indicate that the neoplastic cells are phenotypically helper/inducer T cells.

The claim is further substantiated by the detection of clonal gene rearrangement of the β-chain of the T cell antigen receptor in each biopsy specimen, whereas the immunoglobulin heavy chain genes remained in germline configuration. The intensity of the rearranged bands observed with the TcR_β probe was similar to that in cases of peripheral T cell lymphomas. In contrast to this observation, a mixture of cells from Hodgkin's disease with a large number of epithelioid cells showed only faint rearranged bands resulting from a low percentage (about 5%) of monoclonal proliferating T cells,[25] thus favoring the view that LEL is a T cell lymphoma and not a Hodgkin's disease variant.

In one of the patients, a second lymphoma biopsy was performed 2 years after the first one. Hybridization of the DNA from this second biopsy specimen with the TcR_β probe demonstrated a rearrangement pattern that probably involves three alleles and is completely different from that of the first biopsy specimen. By analogy to B cell lymphomas, a diagnosis of a biclonal T cell lymphoma is in order[26] because upon comparison with the first and the second biopsy samples, the size differences in TcR_β-containing fragments indicate that during the development of the lymphoma other subclones have displaced the original clone. This observation is consistent with the proposal of a multistep pathogenesis as described in acute lymphoblastic leukemias by Fialkow.[27]

In accordance with these findings are the results of cytogenetic studies that have shown numerous chromosomal abnormalities, especially those involving chromosome 3, which is characteristic of T cell lymphomas.[28,29] The characteristically high levels of epithelioid cells in this lesion raise the possibility that the neoplastic CD4-positive clones are functional and produce cytokines to attract these cells.

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von Willebrand's Disease Characterized by Increased Ristocetin Sensitivity and the Presence of All von Willebrand Factor Multimers in Plasma

By Lars Holmberg, Erik Berntorp, Mikael Donnér, and Inga Marie Nilsson

In eight members of one family, platelets in platelet-rich plasma aggregated at much lower ristocetin concentrations than normal. Ivy bleeding time was variously prolonged, and von Willebrand factor antigen (vWF:Ag), ristocetin cofactor activity, and factor VIII coagulant activity were decreased. Most of the affected members had had slight to rather severe bleeding symptoms. Platelet-type von Willebrand's disease (vWD) could be ruled out. All multimers of vWF:Ag were found in plasma as well as platelets. Administration of 1-desamino-8-D-arginine vasopressin (DDAVP) to the propositus did not cause thrombocytopenia, and platelet-poor plasma obtained immediately after did not aggregate normal platelets. The molecular defect in this family, inherited as an autosomal dominant, resembles the one in type IIB because of the response to ristocetin but differs from IIB because all vWF:Ag multimers are present in plasma and the response to DDAVP is atypical. We conclude that this family has a new subtype of vWD and propose that structural as well as functional criteria should be used for a proper classification of vWD.
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VON WILLEBRAND'S DISEASE (vWD) is one of the most common heritable disorders of hemostasis caused by quantitative or qualitative abnormalities of the von Willebrand factor (vWF). Several distinct subtypes exist for which a much-used classification is based on the electrophoretic demonstration of vWF multimeric patterns in plasma and platelets.[1] Broadly speaking, in type I the amount of protein is reduced, but all the multimers are present.[2-4] In type II the electrophoretic appearance of the vWF is qualitatively different, and the various subtypes (II A to D) are characterized by specific abnormalities in vWF multimeric composition.[5-7] Type IIB differs from the other types by also showing an increased interaction between platelets and the vWF in the presence of ristocetin and by having a normal multimeric pattern in platelets, whereas the largest multimers are lacking in plasma.[8] An increased affinity of the IIB vWF for platelets has also been shown in the absence of ristocetin.[9,10] We describe here another subtype of vWD resembling IIB in its increased sensitivity to ristocetin, but having a plasma vWF multimeric composition consistent with type I.

MATERIALS AND METHODS

Blood collection. Blood was collected in a 3.8% trisodium citrate solution at a ratio of 9:1. Platelet-poor plasma (PPP) was prepared by centrifugation at 2,000 g for 20 minutes and platelet-rich plasma (PRP) by centrifugation at 200 g for ten minutes.

1-Deamino-8-D-arginine vasopressin (DDAVP) administration. DDAVP (Ferring, Malmö, Sweden) was diluted in saline and given intravenously (IV) for ten minutes in a dose of 0.4 μg/kg body weight. Blood samples were collected both before the injection and ten minutes after it was completed.

Platelet count. Platelets were counted visually in a phase microscope or electronically using a Coulter counter, model S-Plus II (Coulter Electronics, Hialeah, Fla).

Washed platelets. The platelets in citrated PRP were gently centrifuged (800 g, 30 minutes) onto a cushion of 35% bovine serum albumin (BSA) in 0.01 mol/L phosphate buffer and 0.1 mol/L NaCl, pH 6.6. The platelet layer was aspirated, the platelets being resuspended in the same buffer and washed three times in the same way. Finally, the platelets were resuspended in Tyrode's buffer at a concentration of 4×10^8/mL.

Platelet lysates. One milliliter of PRP with a known platelet count was layered onto a BSA cushion and centrifuged as just described. The supernatant plasma was siphoned off, the albumin mixed with buffer (0.1 mol/L Tris-HCl and 0.15 mol/L NaCl, pH 7.4), and the platelet button spun down and rewashed. To disintegrate the platelets, 100 μL of buffer was added to the button, and the mixture was frozen and thawed five times, after which 5 μL of Triton X-100 (1:5) was added. The platelet cell fragments were spun down at 30,000 g for ten minutes. The amount of von Willebrand factor antigen (vWF:Ag) in the lysate was determined by immunoradiometric assay (IRMA) and further analyzed by multimeric sizing (see the following sections).

Bleeding time. Bleeding time was measured by a modified Ivy technique using a Simplate-II device (General Diagnostics, Morris Plains, NJ).

Factor VIII coagulant activity (VIII:C) and vWF:Ag. VIII:C was measured with a one-stage assay.[11] vWF:Ag was measured both with a quantitative electroimmunoassay (EIA)[12] and IRMA.[13]

Ristocetin cofactor activity (RcoF). RcoF was determined using formalin-fixed platelets as described by Zuzel et al.[14] Ristocetin was obtained from Lundbeck, Copenhagen.

The reference plasma for all assays was citrated plasma pooled from 20 healthy subjects, the pool having been assayed against the 11th British standard for factor VIII (83/509). The values are expressed as units per deciliter.

Crossed immunoelectrophoresis (CIE). CIE was performed in 1% agarose.[15] Results were expressed as ratios between the migrated distances of the peak of the vWF:Ag arc of a sample and that of normal plasma. A ratio greater than 1.2 was considered abnormal.

Ristocetin-induced platelet aggregation (RIPA). RIPA was measured in PRP as described by Ruggeri et al[8] within 30 minutes after blood collection. The ristocetin concentration necessary to induce aggregation with an initial velocity of 20 mm/min was

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extrapolated from the aggregometer tracings at a number of various concentrations. In normal PRP (n = 7) this concentration ranged from 1.00 to 1.70. Thus, aggregation (20 mm/min) occurring at a ristocetin concentration below 1.00 mg/mL was taken as evidence of increased ristocetin sensitivity.

Platelet aggregation. Platelet aggregation with adenosine diphosphate (ADP), epinephrine, and collagen was performed as previously described.[16] Platelet aggregation induced by post-DDAVP plasma was studied as described earlier.[10]

Multimeric sizing. The multimeric distribution of vWF:Ag in PPP and platelet lysates was analyzed by low- and high-resolution sodium dodecyl sulfate (SDS)–agarose electrophoresis (1.9% and 2.5% agarose concentrations, respectively).[17] The bands corresponding to the multimers were identified in the gels by reaction with a ^{125}I-labeled mouse monoclonal antibody followed by autoradiography. This antibody produces multimeric patterns identical to those obtained with rabbit affinity-purified antibodies.[18] Multimeric sizing was performed on samples from patients included in the pedigree (see the section on patients) and from one patient with type IIB vWD in which the largest multimers are known to be lacking in plasma.[9]

Binding of vWF:Ag to platelets. In mixing experiments, washed platelets (normal or patient) were incubated with normal or patient plasma for 15 minutes at room temperature without stirring. Platelets were separated, and residual vWF:Ag was measured in the supernatant by IRMA and expressed as a percentage of the starting value.

Patients. The pedigree is given in Fig 1. The propositus (II:1) is a 46-year-old woman who was referred to our department for preoperative evaluation (hemorrhoidectomy) because of hemorrhagic diathesis. Her history included easy bruising since childhood, subcutaneous hematomas, and gingival bleeding. She has always had trouble with menorrhagia though her two children had been delivered without complications, and she underwent cholecystectomy without abnormal bleeding. The patient was otherwise healthy and was receiving no regular medication, though she treated herself with tranexamic acid (Cyklokapron, 1.5 g three times a day orally) during menstruation periods, which reduced the bleedings. Her 19-year-old son (III:1) had no symptoms, and her 16-year-old daughter (III:2) had easy bruising but no other symptoms. The children's father (II:4) was healthy without bleeding symptoms. One of the patient's sisters (II:2) has had no abnormal bleedings, whereas the other two sisters (II:3 and II:5) and three of their four children reported easy bruising (III:5 to 7). The patient's father (I:6), who died from cancer, never suffered from abnormal bleedings. Her mother (I:5) had easy bruising and menorrhagia. One aunt (I:4) also bruised easily.

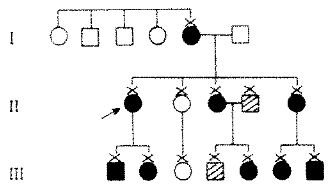

Fig 1. Pedigree of the clinical material. Key: ○, female; □, male; ×, laboratory tests performed; ■, increased ristocetin sensitivity; ▨, normal ristocetin sensitivity, low vWF:Ag concentration. The propositus is indicated by an arrow.

RESULTS

The filled and hatched symbols in Fig 1 indicate family members who are affected according to laboratory values.

The laboratory data of the patients are shown in Table 1. The bleeding time was substantially prolonged in the propositus (II:1) and in III:5 and slightly increased or normal in other family members. The propositus had a slightly decreased vWF:Ag level in plasma, measured by IRMA, and somewhat low RcoF. Other members had slightly decreased, borderline, or normal levels of vWF:Ag and RcoF. On CIE the migration of vWF:Ag was normal in all instances (Fig 2).

When DDAVP was given, the levels of vWF:Ag and VIII:C increased. No major change in the platelet count was seen. The bleeding time was not shortened in the propositus but was normalized in two other family members tested. The post-DDAVP plasma did not aggregate normal platelets in normal PRP (not shown).

In the RIPA test (Table 1 and Fig 3) the platelets from the propositus aggregated at much lower ristocetin concentrations than did normal platelets. Seven other family members also showed greater than normal sensitivity to low ristocetin concentrations in this test. Multimeric sizing of plasma vWF

Table 1. Laboratory Data of the Clinical Material

					vWF:Ag				
						IRMA			
Patient	Sex/Age	Ivy Bleeding Time (Seconds)	Platelet Count (× 10^9/L)	VIII:C (U/dL)	EIA Plasma (U/dL)	Plasma (U/dL)	Platelets (U/10^{11})	RcoF (U/dL)	RIPA test* (mg/mL)
I:5	F/70	600	196	127	114	114	37	108	0.55
II:1	F/47	>1,200 >1,200†	117 131†	46 155†	70 150†	32 117†	22 22†	37	0.73
II:2	F/46	550	169	79	110	114	68	90	1.73
II:3	F/41	740 450†	234 323†	47 193†	50 145†	45 279†	39 51†	51	0.88
II:4	M/42	930	147	83	60	38	10	71	1.10
II:5	F/38	840	185	41	60	37	64	85	0.97
III:1	M/20	840	215	57	64	55	56	46	0.90
III:2	F/16	990	244	74	60	92	60	66	1.12
III:3	F/20	660	286	113	152	152	44	129	1.20
III:4	M/17	630	208	38	34	28	8	38	1.16
III:5	F/15	>1,200 600†	175 160†	83 312†	59 113†	94	17	70	0.90
III:6	F/9	660	201	85	78	80	23	41	0.60
III:7	M/3	780	140	116	100	102	18	117	0.97
Normal controls		360–720	125–340	60–160	50–160	50–175	13–57	50–160	1.00–1.70

*Ristocetin concentration necessary to induce aggregation with an initial velocity of 20 mm/min.

†After DDAVP administration.

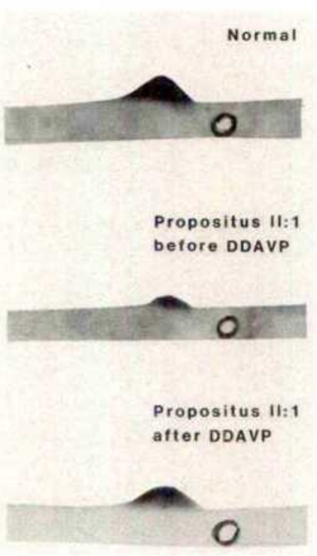

Fig 2. CIE patterns of plasma vWF:Ag from a normal plasma pool and from the propositus (II:1) before and after DDAVP administration. The sample application is indicated by the circled wells. The anode is to the left in the first dimension and to the top in the second dimension.

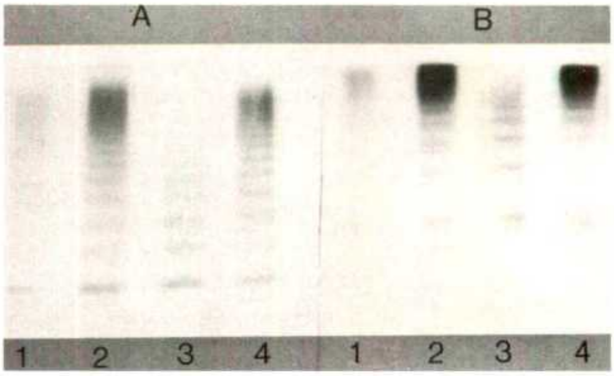

Fig 4. vWF:Ag multimers in plasma analyzed by SDS-agarose gel electrophoresis and identified by reaction with ^{125}I-labeled antibodies followed by autoradiography. Electrophoresis was performed both in 1.9% (A) and in 2.5% (B) agarose gels. Plasma samples from the propositus (II:1) were analyzed before (1) and after (2) DDAVP administration. Control samples from a patient with type IIB vWD (3) and from a normal plasma pool (4) are also shown.

in the propositus (Fig 4) showed all multimers to be present, though in lower than normal concentrations. All multimers were also invariably found in the other members with a heightened ristocetin response. In family member III:4 a quite different result was obtained with a normal RIPA test despite low levels of vWF:Ag and VIII:C. The father of III:4 (II:4), married to a sister of the propositus, also had a low level of vWF:Ag (IRMA) and a normal RIPA test.

The platelet vWF:Ag concentration was normal in all members except II:4 and III:4 in whom it was slightly low. The pattern of vWF multimers in platelets was normal in all.

To study whether the increased platelet aggregation at low ristocetin concentrations was due to an abnormality of the vWF or of the platelets, mixing experiments were performed (see Materials and Methods). Figure 5 demonstrates increased binding of patient vWF:Ag to normal and patient platelets at low ristocetin concentrations. Normal vWF did not show an increased binding to the patient's platelets. Moreover, normal platelet aggregation was found in the propositus when performed with ADP, epinephrine, or collagen. Neither normal plasma nor a factor VIII concentrate (fraction I-0) containing vWF induced aggregation of the patient's platelets.

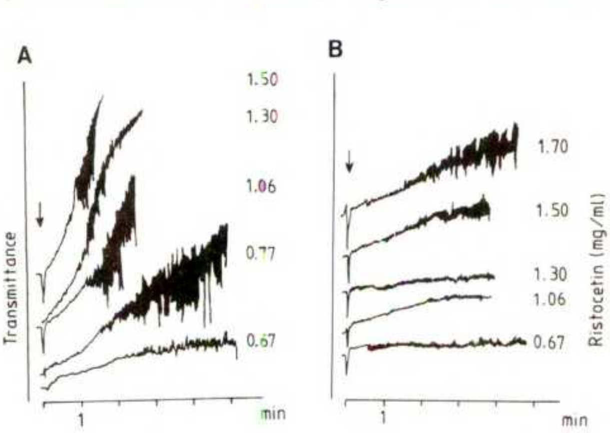

Fig 3. (A) Ristocetin-induced platelet aggregation in PRP from the propositus (II:1). (B) A sample of normal PRP prepared at the same time as the patient's PRP was tested in each experiment.

DISCUSSION

In the family described here the disease differs from all variants of vWD hitherto described. The most conspicuous laboratory finding in the propositus was that platelet aggregation in PRP occurred at much lower ristocetin concentrations than in normal PRP. This indicates an increased interaction between the platelets and the vWF. It was further

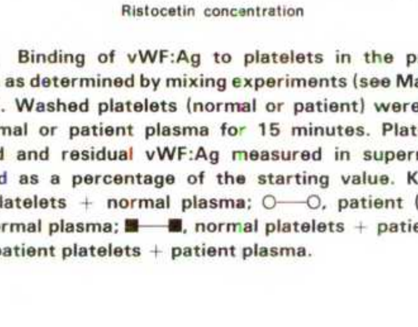

Fig 5. Binding of vWF:Ag to platelets in the presence of ristocetin as determined by mixing experiments (see Materials and Methods). Washed platelets (normal or patient) were incubated with normal or patient plasma for 15 minutes. Platelets were separated and residual vWF:Ag measured in supernatant and expressed as a percentage of the starting value. Key: ●—●, normal platelets + normal plasma; ○—○, patient (II:1) platelets + normal plasma; ■—■, normal platelets + patient plasma; □—□, patient platelets + patient plasma.

demonstrated that the patient's platelets interacted normally with normal vWF and that the abnormality resided in her vWF. An increased interaction between platelets and vWF because of an abnormality in the latter is typical of type IIB vWF[8] and can also be demonstrated in the absence of ristocetin.[9,10,19]

Several findings, however, differentiate the disease described here from type IIB. In IIB, preferentially the large–molecular weight multimers are bound to platelets and thus depleted from plasma, leaving behind the intermediate and low–molecular weight multimers. This is in contrast to the present cases. The normal multimeric pattern of plasma vWF indicates that all molecular forms share the same increased affinity for platelet receptors.[10]

It has recently been shown that patients with type IIB vWD respond to an infusion of DDAVP with thrombocytopenia. This is due to the release by DDAVP of an abnormal vWF with platelet-aggregating properties causing platelet aggregation in vivo.[9] Plasma obtained from IIB patients after DDAVP administration also aggregates platelets in vitro.[9] None of these phenomena occurred in the patients described here. DDAVP infusion did not cause thrombocytopenia, even though the infusion caused a marked increase of vWF:Ag levels; and post-DDAVP plasma did not aggregate platelets in vitro. Thus an interaction between vWF and platelets different from that in type IIB can be postulated also from these findings. Recently some patients have been described demonstrating enhanced RIPA at low ristocetin concentrations, spontaneous platelet aggregation, and thrombocytopenia.[20,21] These patients lacked the high–molecular weight multimers in plasma as in type IIB and may represent one extreme of this variant. They are obviously different from the patients described here.

Type I vWD is characterized by a more or less severe reduction of the vWF in plasma. Typical is the presence in plasma of all vWF multimers, although in reduced amounts, when analyzed by thin-layer SDS–agarose electrophoresis. Different subtypes of type I can be distinguished depending on the relative concentrations of high–molecular weight multimers to lower–molecular weight forms[3] or on the presence in platelets of normal or reduced amounts of vWF[22] or even a qualitatively abnormal platelet vWF.[4] The variant of vWD present in the propositus and other members of her family would be consistent with a type I variant with a normal plasma multimeric pattern and normal platelet vWF. Weiss and Sussman[23] have reported three members of a family who had reduced levels of plasma vWF, with all multimers present, and increased RIPA. Probably our and their families represent a similar vWF trait.

The inheritance of the abnormal ristocetin response in this family obviously follows an autosomal dominant pattern. There was a fairly large scatter in ristocetin sensitivity even among affected members, indicating a variable expression of the genetic trait. This is also reflected in the variable prolongation of the bleeding time from borderline normal to excessively long, in the variability of the clinical picture from rather severe bleeding symptoms to no symptoms at all, and in the variation in factor VIII–associated variables. In addition, it would seem that another abnormal vWF gene is also present in the family. The son (III:4) of one of the propositus's sisters (II:3) had a reduced vWF:Ag level in plasma and platelets but a normal ristocetin sensitivity in the RIPA test. The same was also seen in his father (II:4). The abnormality in these two subjects is consistent with mild type I vWD.

Classification of the various types of vWD has become very intriguing, which is easily understandable in view of the great complexity of the protein involved, the vWF. A large number of mutations of the gene coding for the vWF can be foreseen, leading either to reduced synthesis of the protein, abnormal processing, or structural abnormalities in the amino acid sequence. Thin-layer agarose electrophoresis in SDS[5] has been an invaluable tool in the classification of the various structural abnormalities of vWF. The variant described here shows that this method alone is inadequate for the analysis and classification of vWD. According to multimeric sizing alone, the disease in this family would have been classified as type I vWD, which is unsatisfactory in view of the unequivocal evidence of a functional abnormality of the vWF resembling that in type IIB. Thus both structural and functional criteria are necessary for proper classification. The RIPA test should be used as a regular test for distinguishing variants with an increased vWF-platelet interaction from those with a normal or decreased interaction.

Within the group of increased interaction further subgroups can now be delineated: first, original type IIB patients who respond with platelet aggregation and thrombocytopenia after challenge, eg, with DDAVP and in whom preferentially the large–molecular weight multimers interact with the platelets; second, patients who have spontaneous platelet aggregation, thrombocytopenia, and binding of the high–molecular weight multimers to platelets; and third, patients described here in whom all multimers seem to have the same increased affinity for platelet receptors and in whom thrombocytopenia or platelet aggregation cannot be provoked.

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Effect of Danazol on Clotting Factor Levels, Bleeding Incidence, Factor Infusion Requirements, and Immune Parameters in Hemophilia

By Parvin Saidi, Beatriz Z. Lega, Hugh C. Kim, and Karel Raska, Jr

Several recent studies have reported conflicting results on the effectiveness of danazol, an attenuated androgen, in raising plasma levels of clotting factors VIII and IX in patients with hemophilia. We undertook a randomized, double-blind cross-over trial using 8 weeks' administration of danazol (D), 600 mg/d, and 8 weeks' administration of placebo (P) separated by 2 weeks of rest in 12 patients with hemophilia A and four patients with hemophilia B. Plasma factor VIII and IX levels, frequency and type of bleeding episodes, amount of factor concentrate infused, fibrinogen, fibrinolysis assays, antithrombin III, liver function, and immune parameters were followed. During the danazol phase a minimal increase was noted in the average clotting factor levels, an increase that, although statistically significant, was of hemostatically marginal magnitude. Significant increases in protein C and plasminogen levels, however, were observed during the danazol period, suggestive of danazol-mediated enhanced fibrinolysis. Clinically, bleeding frequency was significantly increased, and more clotting factor was consumed during the danazol period. Furthermore, eight episodes of hematuria and oral mucosal bleeding was reported during the danazol phase in contrast to only one episode of hematuria during the placebo phase, consistent with an enhancement of fibrinolytic activity with danazol. We conclude that danazol does not have a hemostatically significant effect on plasma levels of factor VIII and IX but may be associated with enhancement of fibrinolytic activity, resulting in increased bleeding frequency and requiring more clotting factor infusions. Therefore, danazol is not a viable alternative in the treatment of hemophilia.

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THE AVAILABILITY of clotting factor concentrates has greatly facilitated replacement therapy for the acute bleeding episodes in hemophilia and has vastly improved the general health and life-style of this patient population. There has, however, been a growing concern regarding the potential long-term complications associated with chronic and/or excessive use of such factor concentrates. High incidence of acute hepatitis, chronic liver disease,[1] and more recently human T cell lymphotropic virus Type III (HTLV-III)-positive antibody tests[2,3] are well recognized in patients receiving factor concentrates. Additionally, altered immune status, specifically in the number and function of T lymphocyte subsets, has been reported in hemophiliacs exposed to factor concentrates, suggesting a potential predisposition to the acquired immunodeficiency syndrome (AIDS).[4-8] Therefore, the availability of agents not derived from blood products but capable of increasing plasma levels of clotting factors VIII and IX would be of obvious advantage.

Gralnick and Rick reported in 1983[9] that danazol, an attenuated androgen, administered at doses of 600 mg/d for 14 days to four patients with hemophilia A and one patient with hemophilia B resulted in a significant increase in their plasma clotting factor levels. However, subsequent studies by others did not consistently confirm these findings.[10-13] To further clarify the effect of danazol in a larger number of patients and for a longer duration of treatment, we undertook a double-blind, cross-over study in 22 hemophiliacs treated with danazol or placebo for a period of 8 weeks. Frequency of bleeding episodes, factor concentrate utilization, coagulation and fibrinolysis parameters, and liver function tests were closely followed during danazol and placebo treatment periods. Additionally, since it has been suggested that the action of danazol may, in part, be mediated by an immune mechanism,[14] we followed the immune parameters of our patients during this study.

MATERIALS AND METHODS

Patient population. Twenty-two patients initially consented and entered the study, and 16 patients completed the entire protocol. Five patients were excluded from the study within the initial 4 weeks because of noncompliance to protocol, and one patient was removed from the study because of medical problems unrelated to the protocol. All patients participating in this study were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent.

Sixteen patients completed the entire protocol. Twelve had hemophilia A, six with severe factor VIII deficiency (<1%), three with moderate factor VIII deficiency (1% to 5%), and three with mild factor VIII deficiency (5% to 10%); four patients had hemophilia B, including one with severe factor IX deficiency (<1%) and three with moderate factor IX deficiency (1% to 5%). All patients were older than 18 years of age (mean age, 32 years) and averaged more than six bleeding episodes per year requiring factor infusions. No patient had clinical history or evidence of acute hepatitis within 6 months preceding the study, and no patient had increased levels of inhibitor against clotting factor greater than 1 Bethesda U/mL at any time during the study.

We used a randomized, double-blind cross-over design in which each patient served as his own control. The order in which the drug/placebo was administered was randomly determined. The identity of the drug, which was distributed by the hospital pharmacy, was indicated only by a code number and was known only to the pharmacist. The patients were assigned to start receiving either danazol, 200 mg three times a day, or an identical placebo for a period of 8 weeks; this was followed by 2 weeks of rest, and then they crossed over to the alternate agent. At the end of each phase of the study, a tablet count was done to ensure compliance. Patients kept records of the site and severity of each bleeding episode and the amount of factor concentrate infused. Each patient also completed a questionnaire on the side effects of the treatment at 2, 4, and 8 weeks

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of the treatment period. Coagulation, euglobulin lysis time, and liver function parameters were obtained 1 month prior to the start of the study, days 0 and 3, then weeks 2, 4, and 8 of each treatment period. Protein C, plasminogen, plasminogen activator, and antithrombin III levels were determined at the end of each treatment period. Immune globulin quantitation, complement levels and lymphocyte numbers, and profile and function were determined 1 month prior to the beginning of the study, day 0, and at the end of each treatment period. Skin testing was done prior to the study and at the end of each treatment period.

Laboratory techniques. Blood was drawn through a 19-gauge butterfly from the forearm and placed in polyethylene tubes containing 3.8% sodium citrate (9 vol of blood to 1 vol of anticoagulant). The plasma was immediately separated, rapidly frozen, and stored at −70 °C.[15] The plasma samples were assayed for prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, specific factor assays (factor VIII or factor IX), euglobulin lysis time (ELT), plasminogen, tissue plasminogen activator (t-PA), and protein C at the completion of the study. For tests of coagulation, all samples from each patient were assayed in one batch using the same lots of reagents (deficient plasma, standard reference plasma, and normal pooled plasma) to eliminate the difference inherent in interassays.

Complete blood counts were done by the use of the Coulter Counter (Coulter Electronics, Hialeah, Fla). Liver function tests, including SGOT, SGPT, GGPT (gamma-glutamyl transpeptidase), and alkaline phosphatase were performed in accordance with standard laboratory procedures on the same day that blood samples were obtained.

PT and APTT were performed by the one-stage assay.[16] Fibrinogen levels were assayed based on the thrombin time using Data-Fi Fibrinogen Calibration Reference normal plasma (Dade Diagnostics, Miami).[17] We used the one-stage APTT-based assay for factor VIII and IX with deficient substrate plasma (General Diagnostics, Morris Plains, NJ) and reference normal plasma (Helena Laboratories, Beaumont, Tex). Using three-cycle semilog paper, factor VIII and IX levels were measured as low as 0.5%.[18]

ELT determination was performed with Data-Fi Euglobulin Lysis Reagents for qualitative fibrinolysis testing,[19] and results were expressed as units of activity, with the reciprocal of lysis time of 300 minutes defined as one unit.[20] Plasminogen was determined by the chromogenic substrate method[21] using Spectrozyme PL (American Diagnostica, Greenwich, Conn). Antigenic levels of tissue plasminogen activator was determined by the enzyme-linked immunosorbent assay (ELISA) technique[22] using goat antiserum against human uterine tissue plasminogen activator (American Diagnostica). Protein C was measured by Laurell's rocket immune electrophoresis technique[23] using goat antihuman protein C (American Diagnostica). Antithrombin III was measured by radioimmune diffusion[24] using rabbit antihuman antithrombin III (Behring Diagnostics, La Jolla, Calif).

Peripheral blood mononuclear cells were prepared from heparinized samples by the standard Ficoll-Hypaque gradient.[25] The T lymphocyte populations were quantitated under standard conditions using Ortho-Immune monoclonal antibodies (Ortho Diagnostics, Raritan, NJ).[26] The B cells were quantitated by staining surface immunoglobulins; the natural killer cells were stained with the Leu-7 monoclonal antibody (Becton Dickinson, Sunnyvale, Calif). The immunofluorescence was estimated by flow cytometry in an Ortho System-50 Cytofluorograf.

Mixed lymphocyte reactions (MLR) were carried in round-bottomed microtiter plates using 7.5×10^4 responder and 7.5×10^4 mitomycin C–treated (30 g/mL for 30 minutes) stimulator cells in RPMI 1640 medium supplemented with 10% pooled human serum as described previously.[27] After an 18-hour pulse with ^{3}H-thymidine on the fifth day, the cells were harvested and processed using the automated cell harvester. Phytohemagglutinin (PHA) stimulation was determined with optimal lectin concentrations and 7.5×10^4 mononuclear cells under the same conditions. The level of IgA, IgG, IgM, C3, and C4 were determined in plasma samples kept at −70 °C by radioimmune diffusion[28] using Meloy plates (Meloy Laboratories, Springfield, Calif). Skin tests for delayed sensitivity were performed using three recall antigens; PPD, mumps, and *Candida*. For statistical analysis, the Wilcoxon signed rank test for paired samples (two tailed) was used for comparison between the placebo and danazol phases.

RESULTS

The effects of danazol on clotting factor levels, bleeding frequency, and amount of clotting factor concentrate consumption is shown in Table 1. Of 16 patients evaluated, 12 had hemophilia A, and four had hemophilia B. As a group, the mean factor level during the danazol phase was minimally increased as compared with that of the placebo phase (2.5% ± 1.88% during the danazol phase *v* 2.0% ± 1.96% during the placebo phase, $P = .0002$). Although the incremental increase of mean factor levels for the patients as a group during the danazol phase was statistically significant, its magnitude in terms of hemostatic significance was marginal.

Bleeding frequency was significantly increased during the danazol phase; 53 episodes occurred during the entire 8 weeks of the danazol phase as compared with 33 episodes occurring during the placebo phase, with a mean of 3.0 ± 2.6 episodes per patient in the Danazol phase *v* 1.5 ± 2.4 episodes per patient during the placebo phase ($P = .014$). It is also noteworthy that bleeding sites had a somewhat different pattern during the two treatment periods, with seven episodes of frank hematuria and one episode of spontaneous oral mucosal bleeding reported during the danazol phase in contrast to only one episode of hematuria and no episodes of spontaneous oral mucosal bleeding in the placebo phase. In particular, patient 2, who only bleeds with trauma, experienced increased and spontaneous bleeding episodes during the danazol phase, including one episode of frank hematuria that was controlled with DDAVP infusion. As a result of increased bleeding during the danazol phase, patients consumed more factor concentrate than in the placebo phase (125 ± 135 U/kg/8 wk *v* 71 ± 70 U/kg/8 wk, $P = .024$). In individual patients, there was no clear correlation between bleeding episodes and clotting factor levels. Hematologic parameters (Table 2) revealed no significant differences in WBC counts, fibrinogen, or PT between the placebo and danazol periods. A clinically minimal but statistically significant decrease in the hemoglobin level, increase in platelet counts, and shortening APTT were noted during the danazol period. It is not clear whether these effects are related to increased bleeding episodes or the direct effect of danazol. Antithrombin III levels and fibrinolysis parameters are shown in Table 3. It is of note that protein C, plasminogen, and antithrombin III levels were significantly increased during the danazol period (protein C, 111.12% ± 26.22% *v* 77.62% ± 15.99%, $P = .00003$; plasminogen, 115.33% ± 12.81% *v* 100.25% ± 14.95%, $P = .00003$; antithrombin III,

Table 1. Effect of Danazol on Clotting Factor Levels, Bleeding Frequencies, and Amount of Factor Infusion

Patient	Factor Deficiency	Factor Level* (Percent Activity)		Bleeding Frequency (Episodes/8 wk)		Factor Infusion (U/kg/8 wk)	
		Placebo	Danazol	Placebo	Danazol	Placebo	Danazol
1	VIII	6.0	6.4	0	0	0	0
2	VIII	4.9	5.5	0	3 (H × 1)	0	0†
3	VIII	4.5	4.8	0	0	—‡	—‡
4	VIII	2.6	3.5	8	8	179	255
5	VIII	2.0	2.8	1	1 (H × 1)	43	65
6	VIII	1.8	4.4	2	6	167	314
7	VIII	1.3	1.5	5 (H × 1)	7 (H × 3)	191	477
8	VIII	0.9	0.9	0	4	0	107
9	VIII	0.8	1.6	0	2 (OM × 1)	0	58
10	VIII	0.8	0.6	3	4 (H × 2)	74	150
11	VIII	0.5	0.6	4	3	52	39
12	VIII	0.5	0.5	1	3	52	111
13	IX	1.5	2.5	4	4	140	108
14	IX	1.2	2.1	5	7	73	111
15	IX	1.1	1.3	0	0	28§	0
16	IX	0.9	1.0	2	1	—‖	—‖
Mean ± SD		2.0 ± 1.96	2.5 ± 1.88	1.5 ± 2.4	3.0 ± 2.6	71 ± 70	128 ± 1
P value		.0002		.014		.024	

Abbreviations: H, gross hematuria; OM, bleeding from oral mucosa.

*Factor levels are an average of levels drawn on day 3 and weeks 2, 4, and 8 of each of the placebo or danazol phases. Factor levels drawn within three days of concentrate infusion were not included.

†Patient 2 received five separate infusions of DDAVP to control bleeding during the danazol phase.

‡Amount of factor infusion not included because this patient received a large amount of clotting factor concentrate for major surgical procedure.

§Patient 15 received an infusion of factor concentrate for a dental procedure.

‖This patient's log for factor infusion is not available.

32.5 mg/dL ± 3.8 mg/dL *v* 27.1 mg/dL ± 2.6 mg/dL, $P = 0.001$ during the danazol and placebo periods respectively). t-PA and ELT levels, however, showed no significant differences between the placebo and danazol periods. To rule out the effect of freezing on plasma ELT determination, several samples of normal and abnormal control plasma were tested before and after freezing, and the value of ELT remained unchanged.

Immunologic profiles (Table 4), including total B lymphocyte counts, total T cells measured by OKT3 antibody, T helper cells measured by OKT4, T suppressor subsets measured by OKT8, T4/T8 ratio, natural killer cells, PHA response, and MLR response showed no significant differences between the placebo and danazol phases. However, when these values from both the placebo and danazol phases in hemophilic patients were compared with those of normal healthy volunteers, T4 counts were moderately decreased, and T8 cells were higher than normal, resulting in a reversed T4/T8 ratio. Natural killer cell numbers and the mitogenic lymphocyte response in MLR and after PHA stimulation, although reduced in the hemophiliac, was not affected by danazol treatment. Danazol therapy also did not significantly change the levels of IgA, IgG, IgM, or C3 and C4.

Liver enzymes were followed because danazol is known to adversely affect liver function, but no significant changes from the baseline values were noted during the danazol phase as compared with the placebo phase (Fig 1). Only one patient showed a moderate and transient increase in liver enzymes during both treatment periods, but more pronounced during the placebo phase. No other significant side effects were reported by our patients.

DISCUSSION

An oral agent that could increase factor VIII or factor IX levels in hemophilic patients would offer advantages over the use of clotting factor concentrates derived from pooled plasma with their attendant risk of transmission of donor infections. Gralnick et al[9,29] reported that danazol, an attenuated androgen, is effective in raising plasma levels of factor VIII and IX in patients with hemophilia A and B. However, their observations were based on a very limited number of patients, with variable treatment periods ranging from 2 weeks to 3 months and without parallel of cross-over controls to eliminate placebo effects. Nevertheless, in the context of the current serious concerns regarding blood component

Table 2. Hematologic Profile During Placebo and Danazol Phases in Hemophiliacs

Parameter	Normal Ranges	Placebo	Danazol	*P* Value
Hemoglobin (g/dL)	13.0–16.0	15.2 ± 1.1	14.7 ± 1.0	.02
Platelets ($\times 10^3/\mu L$)	140–440	244 ± 62	278 ± 87	.02
WBC ($\times 10^3/\mu L$)	4.0–10.0	5.5 ± 2.1	5.9 ± 2.1	NS
Fibrinogen (mg/dL)	190–400	228 ± 63	256 ± 92	NS
PT (s)	11.0–13.0	11.4 ± 0.5	11.3 ± 0.6	NS
APTT (s)	25.0–38.0	77.9 ± 17.8	71.8 ± 17.6	.002

Abbreviation: NS, data not statistically significant ($P > .05$).

Table 3. Antithrombin III and Fibrinolysis Parameters During Placebo and Danazol Phases in Hemophiliacs

Parameter	Normal Ranges	Placebo	Danazol	*P* Value
Antithrombin III (mg/dL)	17–30	27.1 ± 2.6	32.5 ± 3.8	.001
Plasminogen (%)	75–120	100.25 ± 14.95	115.31 ± 12.81	.00003
Protein C (%)	60–150	77.62 ± 15.99	111.12 ± 25.22	.00003
t-PA (ng/mL)	12.2 ± 6.1	11.19 ± 6.11	10.11 ± 5.13	NS
ELT (U)	<3	1.15 ± 0.32	1.23 ± 0.31	NS

therapy, we believe their report presented important preliminary observations as a lead to larger controlled studies.

Our results, based on a randomized, double-blind crossover study in 16 patients, showed that mean plasma factor VIII and IX levels during the 8 weeks of danazol treatment were minimally higher for the patients as a group than during the placebo phase. The extent of this increase, however, although statistically significant, was of marginal hemostatic magnitude. In fact, the increased plasma factor levels observed during the danazol phase may, in part, be due to the fact that patients had significantly increased bleeding frequency resulting in higher clotting factor requirements. In three patients who had not had any factor infusion during the entire study (patients 1, 2, and 15), plasma clotting factor levels during the danazol period were only 0.2% to 0.6% higher than during the placebo phase. In contrast, only one patient (patient 6) had a significant incremental increase in his factor level during the danazol phase (4.4%) compared with the placebo phase (1.8%), but this patient had experienced six bleeding episodes requiring factor concentrate infusion with danazol treatment compared with only two such episodes during the placebo period. Alternatively, a danazol-mediated delay in the half-disappearance of infused clotting factor, suggested by Kasper and Boylen,[10] may account for the increase in clotting factor levels for those patients who had significantly increased bleeding episodes requiring factor infusion.

More important, we believe, was the significantly increased bleeding frequency and factor concentrate usage during the danazol period. Furthermore, the danazol phase was associated with an increased incidence of hematuria and bleeding from oral mucosa, suggestive of increased fibrinolytic activity. One patient (patient 2), who is an infrequent bleeder, had three episodes of hematuria during the danazol phase, but none while receiving placebo. Although Gralnick et al[29] reported decreased factor concentrate infusion during danazol therapy, their observations were limited to three patients who received danazol for variable periods. Reports by Kasper and Boylen,[10] Garewal et al,[12] Ambriz et al,[13] and a randomized study by Nugent et al[11] did not find danazol to have consistent or significant effects on raising plasma levels of factor VIII and IX, but remarked on an increase in frequency and/or severity of bleeding episodes in some of the patients while receiving danazol. Enhancement of fibrinolytic activity and increase in protein C levels has been reported with stanozolol,[20,30,31] another anabolic steroid. Similarly, recent reports have suggested that danazol also enhances fibrinolytic activity in hemophilic patients as determined by the euglobulin lysis test.[11,12] In our patients levels of protein C and plasminogen were significantly increased during the danazol phase. Protein C, a vitamin K–dependent plasma protein, when activated by thrombin exerts an anticoagulant effect through inactivation of activated factors V and VIII. Additionally, activated protein C has been shown to enhance fibrinolysis through, as yet, an unknown mechanism.[32,33] A protein C–mediated increase in t-PA has been proposed but not consistently demonstrated in animal models.[33,34] In our patients, no significant difference was noted in t-PA levels between danazol and placebo periods. Although in our patients the ELT showed no change from the baseline during the danazol period, we recognize that ELT is, at best, a crude screening test for plasma fibrinolytic activity. The observed increase in protein C and plasminogen levels during the danazol phase is highly suggestive of danazol-mediated enhanced fibrinolytic activity but needs further substantiation. Furthermore, a direct cause-and-effect relationship

Table 4. Immunologic Profiles During Placebo and Danazol Phases in Hemophiliacs

	Nonhemophilic Controls (N = 25)	Hemophiliacs Placebo (N = 16)	Hemophiliacs Danazol (N = 16)	*P* Value* Controls *v* Hemophiliacs
Total B lymphocytes (/μL)	428 ± 87	249 ± 34	279 ± 71	<.001
Total T lymphocytes (/μL)	1,308 ± 320	1,630 ± 254	1,541 ± 208	<.005
T4 cells (/μL)	863 ± 228	564 ± 144	548 ± 148	<.001
T8 cells (/μL)	512 ± 156	1,050 ± 213	1,017 ± 174	<.001
T4/T8 ratio	1.79 ± 0.54	0.7 ± 0.34	0.68 ± 0.38	<.001
Natural killer cells (%)	10.2 ± 1.3	7.7 ± 2.6	6.9 ± 1.8	<.005
PHA response (× 10^3 cpm)	42.6 ± 13.2	38.1 ± 11.3	41.1 ± 5.4	NS
MLR response (× 10^3 cpm)	2.01 ± 1.11	1.13 ± 4.18	1.30 ± 7.25	NS

**P* values from comparisons between the nonhemophilic controls and the hemophiliacs.

Immunologic profile values are from blood samples drawn during week 8 of both the placebo and danazol phases (N = 16). Nonhemophilic controls were obtained from 25 healthy health professionals of comparable age and sex. No statistically significant difference was noted between the placebo and danazol phase in hemophiliacs.

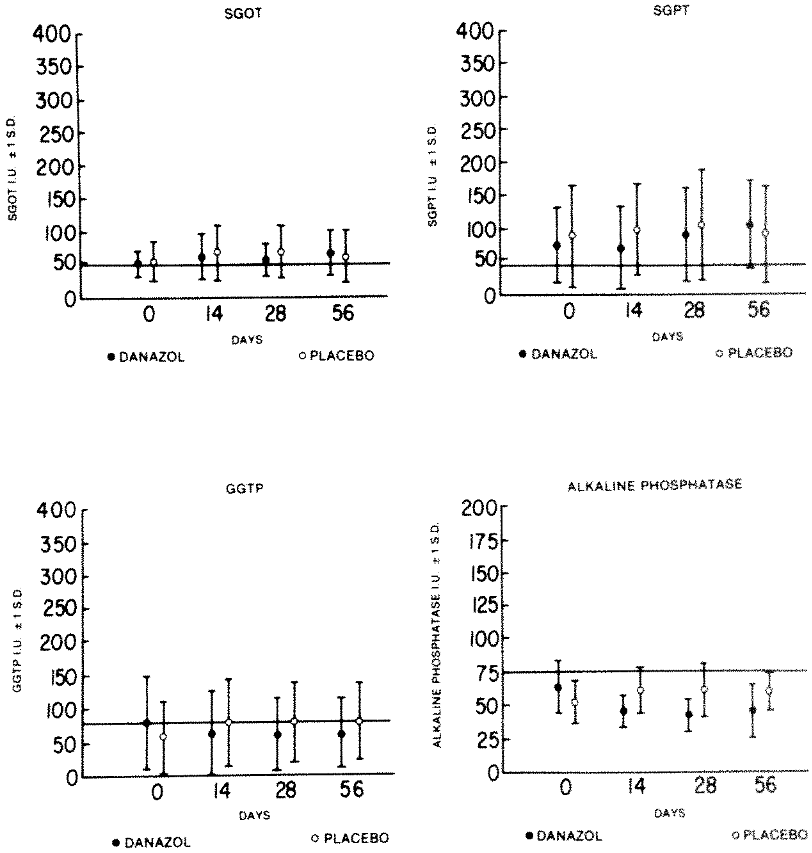

Fig 1. Liver function tests during danazol and placebo periods, including SGOT, SGPT, GGTP, and alkaline phosphatase (mean ± 1 SD). The horizontal bar in each figure represents the upper limit of the normal range.

between enhanced fibrinolysis and the increased bleeding frequency during the danazol phase, although conceptually attractive, remains speculative at this time.

Antithrombin III levels were also significantly increased in our patients during the danazol phase, an observation previously reported with danazol.[35] Antithrombin III and protein C both exert an anticoagulant effect, and increased levels of both would be expected to prolong the APTT. Nevertheless, a shortened APTT has recently been reported in hemophilic patients receiving danazol[11,12] without a consistent parallel increase in factors VIII or IX, and we have also observed a very small, but statistically significant shortening of the APTT during the danazol phase. Garewal et al[36] have suggested that danazol may induce a de novo appearance of an intrinsic coagulation pathway activator bypassing factors VIII and IX. Interestingly, during the danazol phase, there was a decrease in the hemoglobin level and an increase in the platelet count compared with the placebo phase. Danazol has been shown to increase platelet counts in some patients with idiopathic thrombocytopenic purpura (ITP).[37] It is not clear whether in our study increased platelet counts during the danazol phase are due to a similar mechanism as in ITP, possibly immune mediated, or reactive to increased bleeding episodes.

The effect of danazol on the immune system intrigued us. During the last decade, several studies have addressed the effect of androgenic hormones on the immune system, both humoral and cell mediated, in animal models and in patients with autoimmune diseases.[38-41] It has been suggested that danazol may also exert an immune-mediated effect in immune thrombocytopenic purpura.[14,37] We and others had noted abnormalities in T lymphocyte numbers and functions in hemophilic patients.[4-6,42,43] Therefore, we undertook a detailed analysis of humoral and cellular immune parameters in our patients to clarify both the baseline immune status of these hemophilic patients and to document the effect, if any, that danazol had on their immune system. The baseline lymphocyte profiles of our patients were in agreement with earlier reports,[6,42,43] and in general, treatment with danazol produced no significant changes. The total T cell counts remained elevated, and the ratio of functional T cell subsets reversed. The proportion of NK cells enumerated by the Leu-7 antibody remained somewhat depressed. Danazol appears to have no effect on altering this profile. The changed ratio of T cell subsets is reflected by the mitogenic responses with a reduced MLR response. However, the PHA cultures are not significantly affected. Levels of immune globulins IgA, IgG, and IgM and complement components C3 and C4 remained unchanged.

It is of note that liver function parameters did not significantly change during the danazol period, nor were any significant side effects reported by our patients.

Based on our observations, we conclude that danazol, although relatively safe, does not increase plasma levels of factor VIII or IX to a hemostatically significant degree. On the contrary, we and others have observed increased bleeding frequency during danazol therapy. Our data suggest that danazol may enhance the fibrinolytic process through a significant increase in plasma levels of protein C and plasminogen, which may directly or indirectly account for an increase in bleeding frequency and factor concentrate use. Danazol is, therefore, not a viable alternative to factor concentrate infusion in the treatment of hemophilia. Danazol also does not seem to have an effect on the humoral and cellular immune systems as detected by current methodology.

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We wish to acknowledge our gratitude to Winthrop Laboratories for providing the danazol and placebo tablets for this study. We are indebted to Joan Eisele and Linda Zydiak for their nursing care; Maria Propst, Thomas Mathew and Bridget Troccoli for their expert coagulation assays; Debra Donetz and Margaret Montalto for their expert immunological analysis; and Rose Prendergast for the preparation of this manuscript.

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Structural and Functional Differences Between Decay-Accelerating Factor and Red Cell Acetylcholinesterase

By Jeremy Sugarman, Dana V. Devine, and Wendell F. Rosse

The abnormal erythrocytes in paroxysmal nocturnal hemoglobinuria, both PNH II (the moderately abnormal cells) and PNH III (the markedly abnormal cells), lack both acetylcholinesterase (AChE) activity and decay-accelerating factor (DAF) activity. Both of these activities are found on glycoprotein molecules with a molecular weight of about 70 Kd. To demonstrate that these two activities are in fact on different proteins, we have shown that binding to normal red cells of antibody to DAF does not inhibit the subsequent binding of monoclonal antibody to AChE nor AChE activity. Inhibition of DAF activity by polyclonal antibody increases the susceptibility of normal erythrocytes to lysis by complement but inhibition of AChE activity by antibody does not. The rate of decay of the C3 convertase complex of the classical pathway of complement activation was inhibited by DAF added in the fluid phase but not by AChE. When DAF was exhaustively immunoprecipitated from a solution of the erythrocyte membrane proteins, AChE remained and vice versa. These studies indicate that acetylcholinesterase and decay-accelerating factor are two different proteins, both of which are lacking on PNH II and PNH III erythrocytes.

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PAROXYSMAL nocturnal hemoglobinuria (PNH) is a bone marrow disorder characterized by blood cells with an increased sensitivity to lysis by complement.[1-3] The erythrocytes are deficient in red cell acetylcholinesterase (AChE)[4] and this deficiency correlates with the severity of the disease.[5] Since erythrocytes from patients with a congenital, albeit partial, absence of AChE do not manifest an increased susceptibility to lysis by complement,[6] the relationship between the lack of AChE and complement lysis sensitivity in the erythrocytes of patients with PNH is unclear.

The abnormal blood cells in PNH are devoid of decay-accelerating factor (DAF),[7,8] the integral membrane protein described on all cells in the plasma environment[9-11] which modulates both the classical and alternative pathways of the complement cascade.[12] DAF prevents the amplification of the cascade at C3 by interaction with either C4b2a, the classical pathway C3 convertase, or C3bBb, the alternative pathway C3 convertase. The insertion of DAF into the membranes of PNH erythrocytes can partially ameliorate the sensitivity to lysis by complement.[13] Since the DAF defect in PNH parallels the AChE defect, it has been suggested[3] that the two activities reside on the same protein. We performed both functional and immunologic studies to explore the relationship between these two proteins and have demonstrated that the two are different proteins.

MATERIALS AND METHODS

Cells and reagents. For studies using human RBC, blood was collected fresh each day from normal healthy volunteers and anticoagulated in EDTA. Sheep blood was obtained locally and stored in acid citrate dextrose.

Monoclonal antibodies against AChE, AE1, and AE2 were obtained from the American Type Culture Collection (Rockville, Md). A polyclonal antibody to DAF was prepared in rabbits as described in Nicholson-Weller et al.[9] Antiglycophorin activity was removed from the antiserum by absorption against Type III PNH cells which were purified by the method of Chow et al.[14] Normal rabbit serum (NRS) and a mouse monoclonal antibody (P3) with no reactivity on RBC served as controls. Staphylococcal protein A (SPA) was radiolabeled with ^{125}I using Iodogen.[15] Guinea pig C1 and C2 were prepared as previously described.[16]

Effect of anti-DAF or anti-AChE antibody binding on AChE activity. RBC were washed three times in 0.01 mol/L phosphate buffer, pH 8.0, (PB) and resuspended to 1.5×10^9/mL. Antibody binding was performed by incubating one part anti-DAF (1:100), AE-2 (1:400), or nonimmune antibody diluted in phosphate-buffered saline containing 1% bovine serum albumin (PBS-BSA) with two parts washed RBC for 30 minutes at RT. Cells were then washed twice in PBS and diluted 1:600 in PB.

The functional activity of red cell AChE was determined using the method described by Ellman.[17] Briefly, 10 μL of 0.1% quinidine sulfate in absolute ethanol, 25 μL of 0.01 mol/L dithiobisnitrobenzoate (Sigma Chemicals, St. Louis, Mo), and 3 mL of the cell suspension were placed in a cuvette and the reaction was initiated by the addition of 20 μL acetylthiocholine (Sigma). The reaction was monitored at 412 nm for 6 minutes using an LKB Ultrospec which was linked to an Apple II computer with a reaction rate measurement program (Biochrom, Cambridge, England, package 4073-260). The number of moles of substrate hydrolyzed per minute per red blood cell (AU) was determined for each sample according to the equation derived by Ellman.[17]

Competitive binding of anti-DAF and AE2. Washed RBC at 5×10^8/mL were incubated with an equal volume of anti-DAF (1:100) or PBS-BSA for 30 minutes at RT, washed three times in PBS, then resuspended to the starting volume. Each sample was then mixed with an equal volume of AE2 (1:100, 1:200, or 1:400) or PBS-BSA and incubated and washed as described above. To determine binding specificity of the antibodies described above, cells were incubated with NRS or P3 during the first incubation and PBS-BSA in the second. One hundred μL of each sample was then incubated with 100 μL of either ^{125}I-labeled F(ab')$_2$ sheep antimouse immunoglobulin (Amersham, Arlington Heights, Ill) or ^{125}I-labeled SPA for 30 minutes at RT. Three aliquots of 50 μL of each sample were spun through phthalate oils [1.5 parts n-butyl phthalate : 1.0 part bis(2-ethylhexyl) phthalate] to separate bound from unbound radiolabeled probe and the cell pellets counted in a gamma counter. The percent of inhibition of binding was determined by dividing the cpm of the samples incubated with anti-DAF then AE2 by the cpm of the control incubation (NRS then AE2). The experiment was repeated

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reversing the order of antibody incubations. Here, each sample was first incubated with AE2 (1:100), and then with anti-DAF (1:200, 1:500, or 1:1000).

Effect of anti-DAF or anti-AChE antibody on complement lysis sensitivity. A modified version of the complement lysis sensitivity (CLS) test[2] was performed using a total reaction volume of 375 µL, but leaving the proportion of all materials used the same. To determine the effect of DAF or AChE inhibition on the CLS test, cells assayed in the CLS were treated with anti-DAF (1:100), a mixture of AE1 and AE2 (each at 1:100), or nonimmune IgG as described in the competitive binding studies.

Effect of AChE on the decay of C4b2a. Sheep erythrocytes bearing classical pathway C3 convertases (C4b2a) were prepared by reacting sheep RBC suspended in veronal-buffered saline (2.5 mmol/L sodium barbital, 75 mmol/L NaCl, pH 7.5, 0.05% w/v gelatin, 2.5% w/v dextrose, 0.5 mmol/L $MgCl_2$, 0.15 mmol/L $CaCl_2$) (DGVB) with rabbit antisheep erythrocyte antibody prepared in this laboratory, guinea pig C1, and human C4 (Cordis Laboratories, Hialeah, Fla). Guinea pig C2 was added to form sufficient C4b2a to produce approximately 1.0 hemolytic site per cell when the convertase sites were developed with EDTA-chelated guinea pig serum diluted 1:12 in EDTA-veronal buffered saline (5 mmol/L sodium barbital, 0.15 mol/L NaCl, pH 7.5, 0.1% w/v gelatin, 0.04 mol/L EDTA) (EDTA-GPS).

The functional activity of C4b2a was measured using the method of Nicholson-Weller et al[9] for the detection of DAF activity. Red cell AChE (Sigma) was solubilized in DGVB containing 0.01% NP-40. This AChE preparation contained at least 15% AChE as determined by densitometric scan of Coomassie blue staining of the material after SDS-PAGE. EAC142 were incubated with an equal volume of AChE suspension or buffer containing only 0.01% NP-40. Aliquots were removed at various times up to 30 minutes and the residual C4b2a sites developed with EDTA-GPS.

Sequential radioimmunoprecipitation. RBC were radioiodinated as previously described[18] using the Iodogen (Pierce Chemicals; Rockford, Ill) method. Samples containing 1×10^9 ^{125}I-labeled RBC were solubilized in RIP buffer (0.15 mol/L NaCl, 50 mmol/L Tris, 5 mmol/L EDTA, 0.5% NP-40, 0.1% w/v gelatin, pH 7.4) for 30 minutes at RT, and then centrifuged at 4 °C at 15,000 × g for 20 minutes. The supernate was incubated with formalin-fixed *Staphylococcus aureus* strain Cowan I (SAC) (Calbiochem-Behring; La Jolla, Calif) to reduce nonspecific protein binding. After removing the SAC, the supernate was incubated with either 10 µL of anti-DAF or NRS and SAC. Antibody and SAC incubation was repeated four times. Following the last incubation, the supernates were divided in half and incubated with either 20 µL AE2 or P3 or 10 µL anti-DAF or NRS and SAC previously coated with goat antimouse IgG and IgM (Tago; Burlingame, Calif). After washing in SAC buffer all the SAC pellets were boiled in SDS sample buffer to release antigen-antibody complexes. The supernatant fluid was then analyzed by SDS-PAGE (7.5% resolving gel) under nonreducing conditions. The gels were dried and autoradiographed.

Table 1. The Effect of Antibodies to AChE or DAF on the Activity of AChE

Normal RBC Pretreated With	AChE Activity (moles hydrolyzed/min/cell × 10^{-13})
Anti-AChE (AE-2)	1.60
P3 control*	2.21
Rabbit anti-DAF	2.65
Normal rabbit serum	2.08

*Monoclonal antibody lacking reactivity with RBC.

In a similar fashion, a sequential RIP of AChE followed by DAF was performed. Here, the initial precipitation consisted of 20 µL of AE2 or P3 and antimouse-coated SAC. The antibody and antimouse-coated SAC incubations were repeated twice. The resulting supernates were divided in half and incubated with 10 µL of either anti-DAF or normal rabbit serum and SAC. Pellets were prepared for SDS-PAGE and electrophoresis and autoradiography was performed as described above.

RESULTS

The AChE activity of untreated normal RBC was 2.87×10^{-13} moles of substrate hydrolyzed per minute per cell (AU). AChE-deficient PNH Type III cells had an activity of 0.18 AU. Incubation of normal RBC with AE-2 decreased the AChE activity from 2.21 AU to 1.60 AU, while incubation with anti-DAF did not inhibit AChE activity (Table 1).

The binding of anti-DAF to RBC did not inhibit subsequent binding of AE2 at any of the antibody concentrations tested (Table 2). Likewise, binding of AE2 did not inhibit subsequent binding of anti-DAF.

The effect of anti-DAF, AE-1, and AE-2 on the CLS test is shown in Fig 1. Incubation of RBC with two monoclonal antibodies to AChE did not change their sensitivity to complement lysis. In contrast, inhibition of DAF by anti-DAF caused an increased susceptibility to complement lysis.

The rate of decay of the classical pathway convertase from the surface of sheep RBC was not affected by the addition of human red cell AChE to the reaction mixture (Fig 2). This was true at all concentrations of AChE tested up to 10 mg/mL. The decay rates in the presence of 0.01% NP-40 were similar to those measured in the absence of the detergent, however, the overall amount of lysis was slightly greater. This assay readily detected DAF solubilized from the membranes of normal RBC.

As can be seen in Fig 3, all DAF is precipitated from the cell lysate after three incubations in anti-DAF. However, AE2 is still able to precipitate AChE from DAF-free lysate.

Table 2. Competitive Binding of Anti-DAF and Anti-AChE to Normal Erythrocytes

First Incubation	Second Incubation	Third Incubation	% Inhibition of Binding of Second Incubation Antibody
Anti-DAF*	anti-AChE (1:100)	^{125}I-antimouse IgG	4%
Anti-DAF	anti-AChE (1:200)	^{125}I-antimouse IgG	5%
Anti-DAF	anti-AChE (1:400)	^{125}I-antimouse IgG	1%
Anti-AChE†	anti-DAF (1:200)	^{125}I-SPA	0%
Anti-AChE	anti-DAF (1:500)	^{125}I-SPA	6%
Anti-AChE	anti-DAF (1:1000)	^{125}I-SPA	6%

*Anti-DAF was used in the first incubation at 1:100.

†Anti-AChE (AE-2) was used in the first incubation at 1:100.

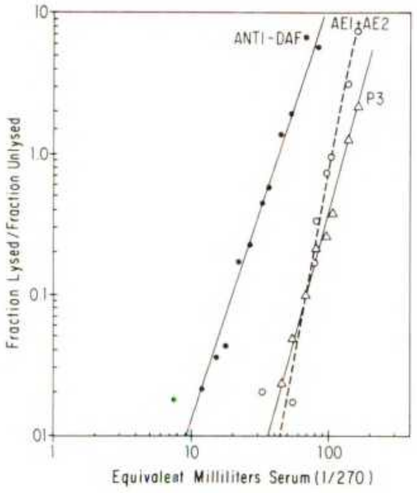

Fig 1. The effect of antibodies to DAF or AChE on the sensitivity of normal erythrocytes to lysis by complement. Cells were pretreated with anti-DAF, monoclonal antibodies to AChE (AE-1 + AE-2), or P3, a monoclonal antibody that does not react with RBC. The cells were then exposed to cold agglutinin anti-I antibody and dilutions of normal human serum as a source of complement. The fraction of cells lysed was determined by spectrophotometric measurement of hemoglobin release and the data analyzed as in Rosse and Dacie.[2] The curve shown for cells treated with P3 is the same as that for cells exposed only to buffer.

Similarly, when all AChE was removed from the lysate by precipitation with AE2 (Fig 4), DAF was precipitated from that cell lysate. Polyclonal anti-DAF precipitated a band with a relative mobility of 63,000 daltons while AE2 precipitated AChE in its dimeric form at 140,000 daltons. Anti-DAF did not precipitate a similar band from PNH erythrocytes (data not shown).

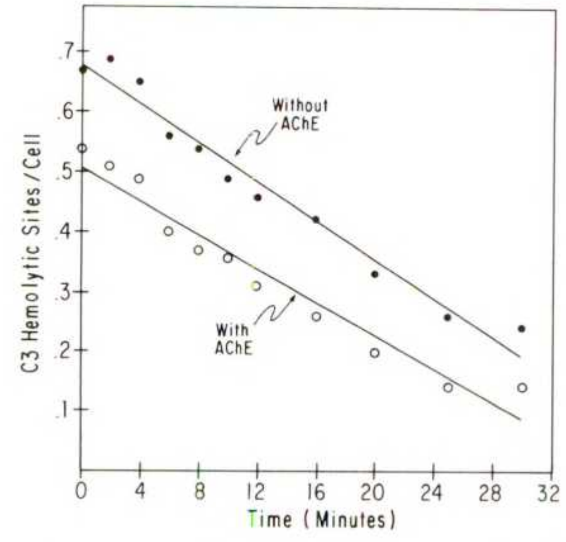

Fig 2. The effect of AChE on the rate of decay of the classical pathway C3 convertase. Sheep erythrocytes bearing C4b2a were incubated in the presence or absence of AChE as described in the text. At various time points, samples were removed and incubated with EDTA-chelated guinea pig serum to measure C4b2a activity. The decay rate of C4b2a was unaffected by the presence of AChE. Slope = −0.01 with AChE and −0.02 without AChE.

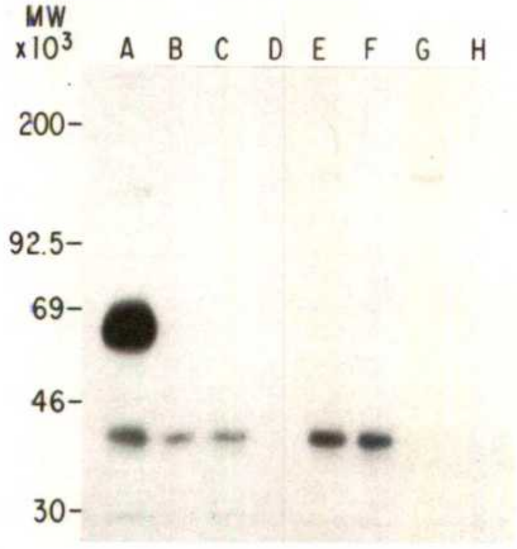

Fig 3. Sequential immunoprecipitation of DAF, then AChE. The first immunoprecipitation with anti-DAF (Lane A) removes a large amount of DAF from the lysate; however, by the final incubation with anti-DAF (Lane C), all DAF has been removed. Lanes B and D are normal rabbit serum (NRS) controls for the first and final precipitations, respectively. AChE was precipitated from the sample which had been previously cleared of DAF (Lane E). AChE appears in its dimeric form under the nonreducing conditions of this gel. AChE was also precipitated from the samples previously incubated with NRS (Lane G). Lanes F and H are immunoprecipitations using the P3 negative control after treatment with anti-DAF or NRS, respectively.

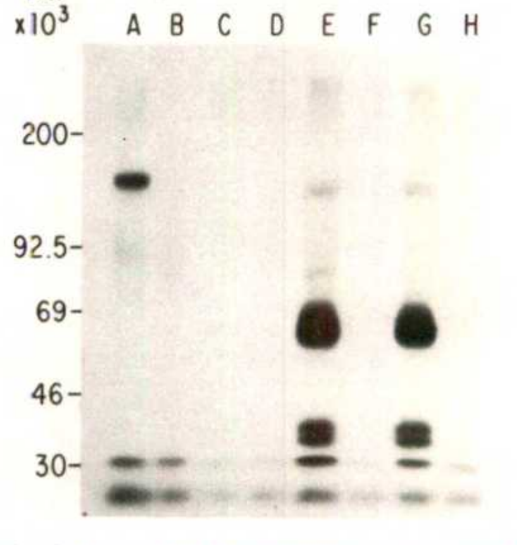

Fig 4. Sequential immunoprecipitation of AChE, then DAF. All AChE was precipitated by monoclonal antibodies to AChE (Lane A) before the final precipitation (Lane C). Once the AChE was removed from the sample, anti-DAF was still able to precipitate DAF from the cell lysate (Lane E). In addition, DAF was precipitated from samples pretreated with the P3 negative control antibody (Lane G). Lanes B, D, F, and H are the nonimmune controls prepared as described in the text for Lanes A, C, E, and G.

DISCUSSION

The abnormal erythrocytes in paroxysmal nocturnal hemoglobinuria are unusually susceptible to the hemolytic action of complement. This is due to an abnormality or abnormalities of the red cell membrane which results in a greater efficiency in the activation of complement on the membrane surface. The deficiency of decay-accelerating factor (DAF), a glycoprotein with a molecular weight of 70 Kd, appears to permit greater activation of C3 on the surface of the cell. However, other abnormalities must be present to account for the marked susceptibility to lysis of PNH III (the most abnormal) cells.

The first membrane abnormality described in PNH was the lack of the activity of the enzyme acetylcholinesterase.[19] Recently, AChE itself has been shown to be absent from the membranes of PNH III erythrocytes.[20] This enzyme has no known function on the cell and its absence or inhibition does not seem to affect the efficiency with which complement is activated. Acetylcholinesterase, like DAF, is about 70 Kd in molecular weight, leading to the suggestion that DAF and AChE activity were in fact resident on the same protein.

The present studies are clear in showing that this is not the case. Inhibition of one activity by antibody does not inhibit the other. The binding of antibody to one does not inhibit the binding of antibody to the other. Immunoprecipitation of one from the solubilized membrane proteins does not remove the other. Finally, inhibition of one (DAF) by antibody alters the effect of complement but inhibition of the other (AChE) does not.

This suggests that the membrane abnormalities in PNH must be complex. The defect is not likely to be due to gene deletion because the precursors of the abnormal erythrocytes appear to possess DAF activity.[21] Rather, the underlying abnormality is probably an alteration in the posttranslational processing or interactions of the membrane proteins. It has been suggested that this might be an abnormality of glycosylation since in some instances the major glycoproteins of the red cell membrane are abnormal[18]; it would be somewhat difficult to account for the total lack of the glycoproteins DAF and AChE from an abnormality in glycosylation unless the abnormality were to destabilize the protein in the membrane.

Whatever the fundamental abnormality is in PNH, it affects at least two distinct membrane proteins, acetylcholinesterase and decay-accelerating factor, of the erythrocytes of patients with the disease. The reason they are both lacking from these cells remains unknown.

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Activation of Human Factor VII in the Initiation of Tissue Factor–Dependent Coagulation

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We have used activation peptide release assays to compare factor VII and activated factor VII (VIIa) activation of factor X, normal factor IX (IX_N), and a variant factor IX (IX_{BmLE}), which, after activation, is unable to back-activate factor VII. In purified systems, factor VII and VIIa each rapidly activated factor X, but after a one minute lag for factor VII. VIIa also readily activated both IX_N and IX_{BmLE}. Factor VII initially failed to activate substantial amounts of either IX_N or IX_{BmLE}; on further incubation factor VII activated IX_N but not IX_{BmLE}. Activation of IX_N began when ∼10% of factor VII had been converted to VIIa, as measured by ^{125}I-factor VII radioactivity profiles. Adding factor VII to VIIa slowed its activation of IX_{BmLE}. However, in the presence of factor X, factor VII alone rapidly activated IX_{BmLE}. Unlike purified systems, 1 nmol/L VIIa added to factor VII-deficient plasma failed to activate factor IX. Increasing factor VII to 10 nmol/L (plasma concentration) either as native VII or VIIa yielded similar activation curves for factor IX and similar activation curves for factor X. Adding 5% VIIa to factor X-deficient plasma and to factor XII-deficient plasma substantially shortened the dilute tissue factor clotting time of only the former. These data support the hypothesis that factor VII/tissue factor complex initiates tissue factor–dependent clotting through a minimal generation of Xa. This Xa then rapidly back-activates a small amount of factor VII, following which the rates of activation of both factors IX and X increase dramatically.

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AFTER exposure of blood to an injured vessel wall a native factor VII/tissue factor complex is formed as the first known step of tissue factor–dependent coagulation. However, native, single-chain factor VII is a zymogen. As measured in in vitro clotting assays, human factor VII activity increases 25- to 30-fold when the native molecule is converted by limited proteolysis to a two-chain activated molecule.[1-5] How such activation of factor VII affects the reactions of tissue factor–dependent coagulation during human hemostasis is as yet incompletely understood. In bovine systems, zymogen factor VII has been shown to have a minimal enzymatic activity[6] thought capable of initiating tissue factor–dependent coagulation. Moreover, bovine factor VII and factor VIIa were found to compete with each other for limited tissue factor sites, and this competition has been postulated to serve as a mechanism for dampening tissue factor–dependent coagulation during hemostasis.[6] However, this hypothesis is difficult to reconcile with the observation that infusion of a low concentration of purified human factor VIIa restored hemostasis in two hemophilia A patients with factor VIII antibodies.[7]

The experiments reported herein were carried out to explore further the roles of human native factor VII and factor VIIa in tissue factor–dependent activation of factors X and IX in both purified and plasma systems. The availability of a purified variant factor IX molecule, factor IX_{BmLE}, that cannot back-activate factor VII[8] allowed us to examine critically the relative efficiencies of native factor VII and factor VIIa as activators of factor IX over a prolonged reaction period. It also facilitated testing the hypothesis of competition between human native factor VII and factor VIIa as a regulatory mechanism for tissue factor–dependent coagulation.

MATERIALS AND METHODS

Reagents. Tissue factor was a saline extract of human brain tissue. It clotted normal pooled plasma in 13 seconds in a plastic tube. Hereditary clotting factor–deficient plasmas were obtained from George King Bio-Medical (Overland Park, Kan) or from patients seen in this laboratory. Rabbit brain cephalin was obtained from Sigma Chemical Co (St. Louis, Mo). Sodium ^{125}[I] and Na^3[H]borohydride were purchased from Amersham Corp (Arlington Heights, Ill). Unless otherwise specified all reagents were diluted in a buffer (TBS/BSA) containing 0.05 mol/L Tris. HCl, 0.15 mol/L NaCl, pH 7.5, and 1 mg/mL bovine serum albumin.

Purified human clotting factors. Human factor VII,[9] factor IX,[10] and factor X[10] were purified to homogeneity as previously described. Factor IX_{BmLE} was purified in this laboratory using the same technique as for normal factor IX. Its properties have been described in detail.[8]

Factor Xa was prepared by incubating purified factor X with insolubilized Russell's viper venom in the presence of calcium.[10] It was stored at −20 °C in 50% v/v glycerol and diluted to the desired concentration in TBS/BSA just prior to use. The fully activated factor Xa (as documented by SDS-polyacrylamide gel electrophoresis) had a specific activity, as measured in our factor Xa assay,[5] of 1,245 U/mg.

Factor VIIa was prepared by incubating factor VII (2.5 ug/mL) with factor Xa (0.1 ug/mL) in the presence of cephalin (1% v/v) and 6 mmol/L calcium for 15 minutes at 37 °C. The reaction was terminated by adding 12 mmol/L EDTA. For most experiments factor VIIa preparations were incubated with antibodies to factor X for 20 minutes at room temperature to neutralize the trace amounts of factor Xa present in the preparations. Full neutralization of

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residual factor Xa by the anti–factor X antibodies was established by showing failure of cleavage of ^{125}I-factor VII added to preparations after the antibodies. For the experiments on the clotting times of factors X- and XII–deficient plasmas, factor VIIa was prepared at a final concentration of 150 ug/mL using 3.0 ug/mL factor Xa, 0.42 mmol/L cephalin, and 5 mmol/L Ca^{2+}, and diluted in the experiment to a final concentration of 25 ng/mL.

Concentrations of purified clotting factors were determined spectrometrically at A 280 nm using E_{cm} 1% of 13.9 for factor VII,[3] 13.2 for factor IX_N and factor IX_{BmLE},[11] and 11.6 for factor X.[11]

Radiolabeling of proteins. Sialyl ^{3}H-normal factor IX (IX_N) and sialyl ^{3}H-factor IX_{BmLE} were prepared by the general technique of VanLenten and Ashwell[12] and as described earlier.[13] Specific radioactivity for ^{3}H-factor IX_N was 3.1×10^8 CPM/mg and for ^{3}H-factor IX_{BmLE} was 4×10^8 CPM/mg protein. Sialyl ^{3}H-factor X was also prepared by the same method with a slight modification as previously described.[5] Its specific activity was 1.3×10^9 CPM/mg. Radiolabeled preparations had from 80% to 90% of the biologic activity of nonlabeled preparations as measured in one-stage clotting assays.[10] Factor VII was labeled with ^{125}I as described earlier.[5]

Preparation of antibodies. An anti–factor X antiserum was prepared as described earlier.[5] The antiserum was adsorbed with barium sulfate, heated to 56 °C for 20 minutes and then subjected to DEAE-Affi Gel Blue chromatography to obtain the IgG fraction. It gave a single precipitation line of identity on double immunodiffusion plates with purified factor X and normal plasma. A 100-fold dilution of the IgG fraction of this antiserum neutralized 60% of the factor X clotting activity in normal plasma. Adding the antibody to a reaction mixture of factor Xa, phospholipid, calcium ions, and factor VII prevented any measurable activation of the factor VII by factor Xa.

Activation peptide release assays. Rates of factor IX and factor X activation were monitored as described previously.[14,15] One hundred percent activation of factor IX or factor X yielded approximately 35% to 40% trichloroacetic acid soluble counts.

Measurement of activation of ^{125}I-factor VII. Activation of ^{125}I-factor VII added to reaction mixtures was measured by subjecting serial aliquots from mixtures to reduced SDS polyacrylamide gel electrophoresis and analyzing the resultant radioactivity profiles. The method has been described in detail earlier.[5]

RESULTS

Activation of factor IX_N and factor IX_{BmLE} in a purified system by factor VIIa/TF and by factor VII/TF. Factor VIIa/TF activated ^{3}H-factor IX_N and ^{3}H-factor IX_{BmLE} at similar initial rates in reaction mixtures containing 1 nmol/L factor VIIa, 10% (v/v) tissue factor, 5 mmol/L calcium, and 88 nmol/L substrate (Fig 1). Progress curves differed only in that activation of ^{3}H-factor IX_{BmLE} reached a plateau at a lower release of TCA soluble counts than did activation of factor IX_N. To explore this difference further, we subjected samples from activation mixtures to SDS-polyacrylamide gel electrophoresis and measured percent activation from the ratio of counts eluted from the activation peptide peak to total counts eluted from the gel. When measured by this technique factor VIIa/TF activated approximately 80% of both ^{3}H-factor IX_N and ^{3}H-factor IX_{BmLE} after one hour. Thus, as suspected from their virtually identical initial rates of activation (see Fig 1), the lower plateau for the activation peptide release assay for ^{3}H-factor IX_{BmLE} (24% TCA soluble counts) than for ^{3}H-factor IX_N (32% TCA soluble counts) did not reflect a substantial difference in the behavior of these two substrates toward activation by factor VIIa/TF.

Very different data (Fig 1) were obtained when native factor VII was substituted for factor VIIa. In these experiments antibodies to factor X (2% v/v) were added to the reaction mixtures to neutralize any traces of factor X contaminating our reagents. Under these conditions only barely measurable TCA soluble counts were released within the first 20 minutes from either ^{3}H-factor IX_N or ^{3}H-factor IX_{BmLE}. However, after 20 minutes the rate of release of TCA soluble counts from ^{3}H-factor IX_N increased strikingly and approached that obtained with factor VIIa/TF. In contrast, the release of TCA soluble counts from ^{3}H-factor IX_{BmLE} did not increase, a reflection of the inability of activated factor IX_{BmLE} to back-activate factor VII. Even after two hours of incubation, only 2.5% TCA soluble counts were released from ^{3}H-factor IX_{BmLE}.

Our reaction mixtures apparently contained otherwise undetectable traces of factor X or factor Xa since activation of factor IX_{BmLE} was measurable after 20 minutes of incubation and was appreciable by 60 minutes of incubation in reaction mixtures containing factor VII in which antibodies to factor X were left out. Therefore, the very minimal evidence of activation of factor IX_{BmLE}—2.5% release of TCA soluble counts at two hours—in reaction mixtures with factor VII–containing antibodies to factor X could reflect formation of minute amounts of factor VIIa catalyzed by minute amounts of free factor Xa in the incubation mixture despite the presence of the antibodies to factor X.

Activation of factor X in a purified system by factor VIIa/TF and by factor VII/TF. Factor X was activated effectively in reaction mixtures containing either 0.25 nmol/L factor VII or 0.25 nmol/L factor VIIa, 5% (v/v) TF, 5 mmol/L calcium, and 175 nmol/L ^{3}H-factor X (Fig 2). However, progress curves differed in that a lag period of

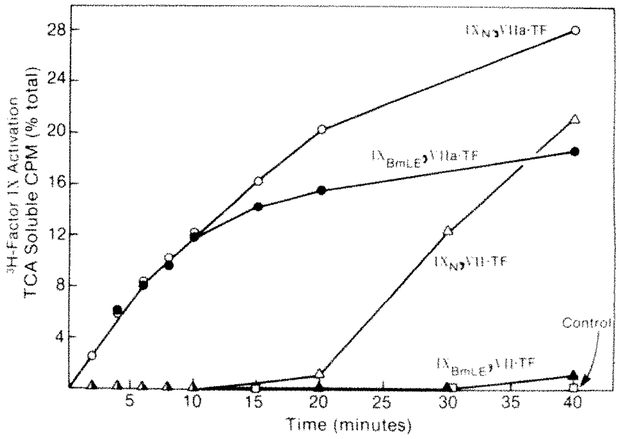


Fig 1. Activation of factor IX_N and factor IX_{BmLE} by factor VII/TF and by factor VIIa/TF in a purified system. Final concentrations: ^{3}H-factor IX_N or ^{3}H-factor IX_{BmLE}, 88 nmol/L; factor VII or VIIa, 1.0 nmol/L, tissue factor, 10% v/v; Ca^{2+}, 5 mmol/L and anti–factor X antibodies, 2% v/v. Neither factor VII nor tissue factor were added to ^{3}H-factor IX_N in the control reaction mixture (□). Other symbols are: (○) IX_N, VIIa/TF; (△) IX_N, VII/TF; (●) IX_{BmLE}, VIIa/TF; (▲) IX_{BmLE}, VII/TF.

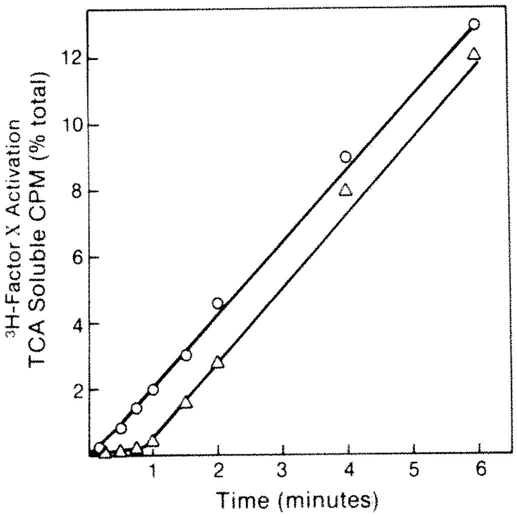


Fig 2. Activation of factor X in a purified system by factor VIIa/TF and by factor VII/TF. Final concentrations are: ^{3}H-factor X, 175 nmol/L; factor VII or factor VIIa, 0.25 nmol/L; tissue factor, 5% v/v; and Ca^{2+}, 5 mmol/L. Symbols are: (○) VIIa/TF; (△), VII/TF.

about one minute elapsed before measurable TCA soluble counts were released from a reaction mixture containing native factor VII. Then, both reaction mixtures yielded the same maximal rate of release of TCA soluble counts. This suggests to us that native factor VII is also a poor activator of factor X. Yet, enough factor Xa was either present as a contaminant or was quickly formed after recalcification to generate sufficient VIIa to yield an inflection point after only one minute.

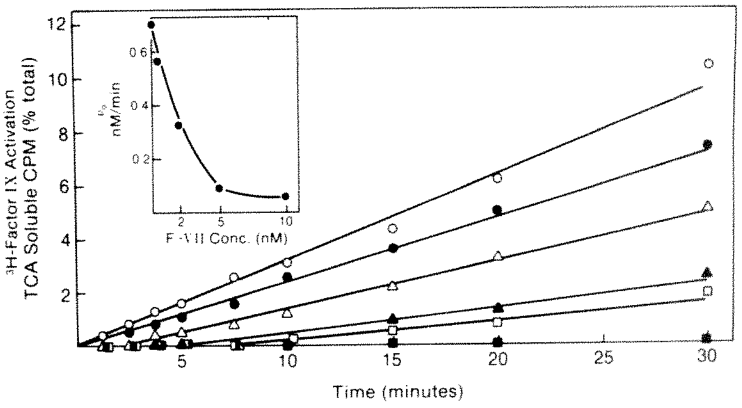
Lacking a variant factor X molecule with properties analagous to factor IX_{BmLE}, we were unable to study the ability of native factor VII to activate factor X over a prolonged period. We treated normal factor X with succinic anhydride or with citraconic anhydride in the hope of preparing a molecule that could be activated normally but would fail to back-activate factor VII. However, although a modified factor X was prepared that failed to convert measurable amounts of prothrombin to thrombin in the absence of factor V, the modified molecule retained partial ability to activate factor VII. We also carried out experiments in which the active site inhibitor, dansyl-glu-gly-arg-chloromethylketone, was added to our factor X and factor VII reagents but the resultant data were inconclusive.

Competitive inhibition of factor VIIa activity by native factor VII in purified systems. In a bovine purified system, factor VII can inhibit factor VIIa catalyzed activation of factor IX.[6] Since factor IXa_{BmLE} cannot activate factor VII, factor IX_{BmLE} is an ideal substrate to examine such inhibition in a human purified system. Reaction mixtures were prepared containing a constant concentration of factor VIIa (0.5 nmol/L), a low, limiting concentration of TF (2.5% v/v), and increasing concentrations of native factor VII (from none to 10 nmol/L). As shown in Fig 3, increasing the concentration of native factor VII progressively decreased the rate of activation of factor IX_{BmLE} (eg, from 0.7 nmol/L/min with no native factor VII to 0.1 nmol/L/min with 5 nmol/L native factor VII). Similar data were obtained for the activation of normal factor IX except that after an interval of about 15 minutes the initial rates increased due to back activation of factor VII by the initial factor IXa generated in the reaction mixture.

The effect of factor X upon native factor VII/tissue factor activation of factor IX_{BmLE}. Adding a plasma concentration of factor X (140 nmol/L) to reaction mixtures abolished the striking difference observed between the rate of activation of ^{3}H-factor IX_{BmLE} by factor VII and the rate of activation of ^{3}H-factor IX_{BmLE} by factor VIIa. As shown in Fig 4, after a one-minute lag period, a reaction mixture containing factor VII and a reaction mixture containing factor VIIa had similar rates of activation. The added factor X influenced only the activation of factor IX_{BmLE} by native factor VII and not the activation of factor IX_{BmLE} by factor VIIa. One may infer from the data of Fig 4 that native factor VII cannot function as a meaningful regulator of factor VIIa activity in purified reaction mixtures containing factor X.

The relation between activation of factor VII and activation of factor IX in reaction mixtures. The data of Fig 1 were interpreted to mean that accumulation of a very low concentration of factor IXa in reaction mixtures with native factor VII can lead over time to the generation of enough factor VIIa to increase markedly subsequent activation of factor IXa. Additional experiments were carried out to determine how much factor VIIa was present at the inflec-

Fig 3. The effect of native factor VII upon the rate of factor VIIa/TF catalyzed activation of factor IX_{BmLE} in the presence of a limiting concentration of tissue factor. Final concentrations: ^{3}H-factor IX_{BmLE}, 88 nmol/L; TF, 2.5%, v/v; Ca^{2+}, 5 mmol/L. The symbol (○) identifies a reaction mixture containing 0.5 nmol/L factor VIIa; the symbol (■) identifies a reaction mixture containing 10 nmol/L factor VII. The other symbols denote reaction mixtures containing 0.5 nmol/L factor VIIa plus increasing amounts of factor VII as follows: (●) 0.5 nmol/L; (△) 2.0 nmol/L; (▲) 5 nmol/L; (□) 10 nmol/L. The insert is a secondary plot of initial rate of activation of factor IX_{BmLE} against concentration of native factor VII added to factor VIIa.



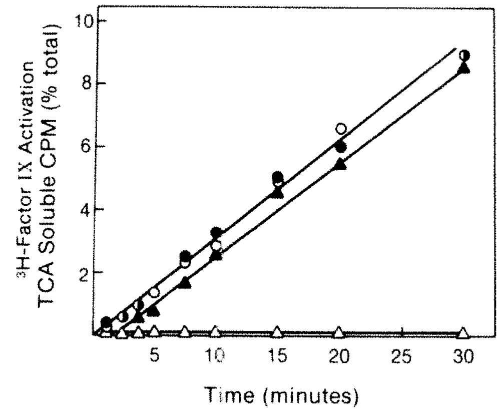


Fig 4. Activation of factor IX_{8mLE} by factor VII/tissue factor or factor VIIa/tissue factor in the presence or absence of factor X. All reaction mixtures contained ^{3}H-factor IX_{8mLE}, 88 nmol/L; TF, 2.5% v/v; and Ca^{2+}, 5 mmol/L. Other reagents were: (○), 0.5 nmol/L VIIa; (●) 0.5 nmol/L VIIa plus 140 nmol/L factor X; (▲), 10 nmol/L factor VII plus 140 nmol/L factor X; (△) 10 nmol/L factor VII.

tion point of factor IX activation in such reaction mixtures. On two occasions paired reaction mixtures were prepared. In one reaction mixture containing ^{3}H-factor IX and nonlabeled factor VII, the rate of activation of factor IX was measured by activation peptide release assay. In the second reaction mixture, containing nonlabeled factor IX and ^{125}I–factor VII, the rate of activation of factor VII was measured from radioactivity profiles. Experimental conditions differed from the experiments of Fig 1 in that the factor VII concentration of reaction mixtures was increased from 1 nmol/L to 10 nmol/L in order to obtain sufficient counts for the radioactivity profiles. Thus, in these experiments both factor VII and factor IX were at plasma concentrations.

Data are summarized in Fig 5. Activation of factor IX in the reaction mixture made with factor VII was clearly measurable at 15 minutes, a time when only about 5% of the factor VII had been activated to factor VIIa. The highest rate of activation of factor IX was reached by 25 minutes, at which time about 25% of factor VII had been converted to factor VIIa. Note, however, that the final rate of activation of factor IX in the mixture with factor VII was not as rapid as the initial rate of activation of factor IX in a control mixture containing only preformed factor VIIa. This slower rate could reflect a possible effect of competition of factor VII and factor VIIa for limited tissue factor sites, or an effect of substrate depletion, or both.

Since the lag period before the inflection point in progress curves of native factor VII activation of factor X was only one minute, we did not attempt to obtain similar data for the activation of factor X.

Activation of ^{3}H-factor IX and ^{3}H-factor X by factor VII/TF and by factor VIIa/TF in plasma systems. Reaction mixtures were made containing factor VII–deficient plasma (75% v/v) or buffer (control), 10% (v/v) TF, 88 nmol/L ^{3}H-factor IX_N, 10 mmol/L calcium, and 1 nmol/L factor VIIa. Thus, factor VIIa was present at one tenth the normal plasma concentration of factor VII. As shown in Fig 6, whereas ^{3}H-factor IX was activated effectively in the control system without added factor VII–deficient plasma (25% TCA soluble counts released), ^{3}H-factor IX was activated only minimally in the system containing plasma (about 1% TCA soluble counts released). Increasing the TF concentration to 20% (v/v) in mixtures containing plasma did not

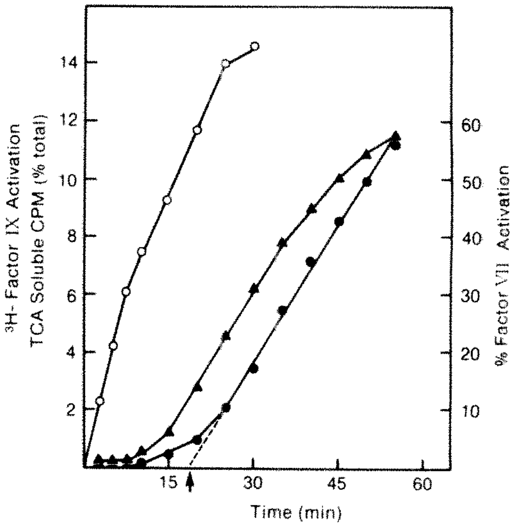


Fig 5. The relation between activation of factor IX and activation of factor VII in reaction mixtures containing native factor VII and factor IX. All reaction mixtures contained factor IX_N, 88 nmol/L; TF, 10% v/v; anti–factor X antibodies, 2% v/v; Ca^{2+}, 10 mmol/L. The symbol (○) identifies a reaction mixture containing nonlabeled VIIa, 10 nmol/L, and ^{3}H-factor IX_N. The symbol (●) identifies a reaction mixture containing nonlabeled factor VII, 10 nmol/L, and ^{3}H-factor IX_N. The symbol (▲) identifies a reaction mixture containing ^{125}I-factor VII, 10 nmol/L, and nonlabeled factor IX_N. The arrow mark indicates lag period of 18.5 minutes for activation of factor IX with native factor VII.

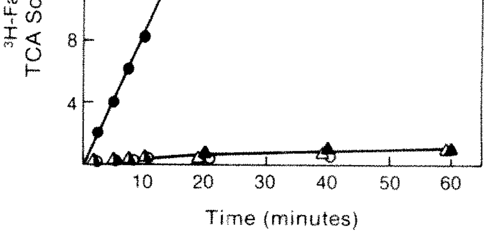


Fig 6. The failure of 1 nmol/L factor VIIa/TF to activate factor IX_N in factor VII–deficient plasma. Final concentrations are: factor VII–deficient plasma or control buffer (●), 75% v/v; VIIa, 1 nmol/L; ^{3}H-factor IX_N, 88 nmol/L; Ca^{2+}, 10 mmol/L. Final concentrations of tissue factor, v/v varied as follows: (○, ●), 10%; (△) 15%, (▲), 20%.

increase the extent of activation. These data reflect the potent ability of a plasma inhibitory mechanism recently described from this laboratory[16] to inhibit the enzymatic activity of factor VIIa/tissue factor.

Since 1 nmol/L factor VIIa activated factor IX only minimally in factor VII–deficient plasma, it was pointless to look for a dampening effect of added native factor VII at this factor VIIa concentration. Therefore, in additional experiments factor VII–deficient plasma was supplemented with either native factor VII or factor VIIa to a 10 nmol/L concentration (normal plasma factor VII concentration) and the course of activation of added ^{3}H-factor IX or added ^{3}H-factor X was measured. (In these experiments a 5% (v/v) tissue factor concentration sufficed to release substantial TCA soluble counts from ^{3}H-factor X but a 20% (v/v) concentration of tissue factor was required to obtain readily measurable TCA soluble counts from ^{3}H-factor IX.) As shown in Fig 7, native factor VII and factor VIIa gave similar curves of activation for factor IX and similar curves of activation for factor X. However, because of delay due to removing fibrin from reaction mixtures, the first data point was obtained 2.5 minutes from recalcification. Thus a transient lag in reaction mixtures containing native factor VII would have been missed.

Effect of factor VIIa upon clotting times of factor XII–deficient plasma and factor X–deficient plasma. Since a 1 nmol/L concentration of factor VIIa did not activate measurable amounts of factor IX in plasma (Fig 6) and since a 10 nmol/L concentration of factor VIIa or factor VII yielded similar rates of activation for both factor IX and factor X in plasma (Fig 7), we turned next to clotting time systems. The effect was examined of a low concentration of factor VIIa (5% of normal factor VII plasma concentration) upon the clotting time with dilute tissue factor of factor XII–deficient plasma and of factor X–deficient plasma. The added factor VIIa had an insignificant effect upon the clotting time of factor XII–deficient plasma (eg, shortening of the clotting time from 180 seconds in its absence to 150 seconds in its presence). In contrast, the added factor VIIa had an appreciable effect upon the clotting time of factor X–deficient plasma (eg, shortening of the clotting time from 270 seconds in its absence to 148 seconds in its presence).

DISCUSSION

The availability of purified factor IX_{BmLE}, which like normal factor IX is readily activated by factor VIIa/tissue factor but which, unlike normal factor IX, cannot back-activate factor VII—enabled us to examine critically the ability of *native* factor VII/tissue factor to activate factor IX. Native factor VII/tissue factor was found to be essentially unable to activate factor IX_{BmLE}; only traces of factor IX_{BmLE} were activated (2.5%) after 40 minutes of incubation in a purified system (Fig 1). Therefore, we believe that native factor VII/tissue factor activation of normal factor IX probably does not function as a physiologically significant trigger mechanism for initiating tissue factor–dependent coagulation during hemostasis.

However, our data do not permit us to conclude that native factor VII/tissue factor is completely inert toward factor IX in human systems. Zur et al[6] and Jesty and Morrison[17] have presented evidence that native bovine factor VII possesses a minimal enzymatic activity. The trace activation of factor IX_{BmLE} that we observed after 40 minutes could reflect activation by native factor VII/tissue factor—or could reflect activation by otherwise unmeasurable traces of either preformed factor VIIa in our reagents or of factor VIIa activated by a minute amount of factor Xa generated in reaction mixtures despite the presence of antibodies to factor X.

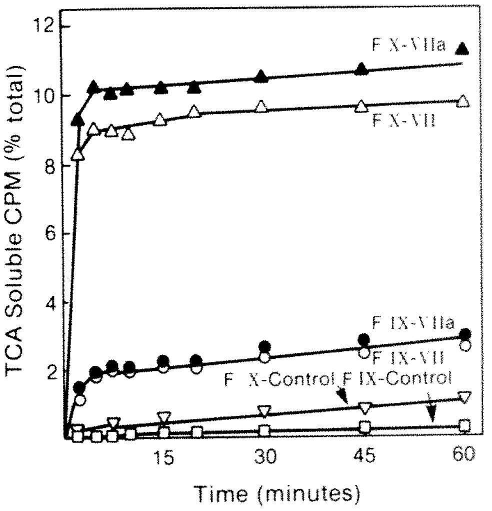


Fig 7. Activation of factor IX and factor X by 10 nmol/L factor VII/TF or 10 nmol/L VIIa/TF in factor VII–deficient plasma. Final concentrations are: factor VII–deficient plasma, 75% v/v; VIIa or factor VII, 10 nmol/L; ^{3}H-factor X, 175 nmol/L, or ^{3}H-factor IX$_N$, 88 nmol/L; TF, 5% v/v in mixtures containing ^{3}H-factor X and 20% in mixtures containing ^{3}H-factor IX; Ca^{2+}, 10 mmol/L. Control mixtures contained no added factor VII or VIIa. Symbols are: (▲) VIIa and factor X; (△) VII and factor X; (●) VIIa and factor IX; (○) VII and factor IX; (▽) factor X control; (□) factor IX control.

Because we could not prevent back-activation of factor VII by factor Xa, we could not obtain critical data on the rate at which native factor VII can activate factor X over a prolonged reaction period in purified human systems. The data for the first minute of the progress curves of factor X activation (Fig 2) suggest that native factor VII also functions as a poor activator of this substrate. The inflection point at one minute undoubtedly reflects back activation of factor VII by a trace amount of factor Xa, which in the presence of phospholipid and calcium ions is a highly efficient activator of factor VII.[4,17-19] As already mentioned for factor IX_{BmLE}, this factor Xa could have been generated either by the catalytic effect of native factor VII or by contaminating factor VIIa in our reagents.

When purified reaction mixtures (Fig 4) or plasma systems (Fig 7) contained native factor VII and both factor IX and factor X, activation of factor IX was noted after only a minimal lag period. This presumably reflected an initial generation of traces of factor Xa with resultant prompt formation of factor VIIa. From the data in purified systems of Fig 5, it appears that less than 10% of the plasma concentration of factor VII needs to be converted to factor VIIa to initiate significant activation of factor IX at its plasma concentration. These data fit with earlier observations of others,[17,19,20] which suggest that generation of small amounts of factor VIIa induced by adding a low concentration of factor Xa to bovine and human plasma systems initiates the effective generation of factor IXa. Therefore, although *native* factor VII/tissue factor activation of factor IX may not represent a physiologic initiation mechanism for tissue factor–dependent coagulation, factor VIIa/tissue factor activation of factor IX could still play a physiologically meaningful role in tissue factor–dependent coagulation during hemostasis.

The ability of a minimal concentration of factor Xa to activate factor VII rapidly in plasma also can account for the different effects we observed on adding a low concentration of factor VIIa to factor XII–deficient plasma and to factor X–deficient plasma. In the former, a normal concentration of factor X presumably made possible the ready generation of traces of factor Xa and therefore minimized the effect of added preformed factor VIIa. In the latter, a concentration of factor X of less than 1% of normal plasma concentration, greatly impaired the generation of a trace of factor Xa, and brought out the effect of added preformed factor VIIa.

Zur et al[6] showed in a bovine system that factor VII could compete with factor VIIa for limited tissue factor sites and suggested that such competition could function as a form of control of coagulation with implications for hemostasis. We have now confirmed their observation in a human system with factor IX_{BmLE} (Fig 3). However, the ability of factor VII to activate factor IX_{BmLE} readily after a one-minute lag period in reaction mixtures containing factor X (Fig 4) means to us that traces of factor Xa can rapidly activate factor VII complexed with tissue factor in purified systems. This abolishes any competitive effect of native factor VII in such systems.

We could not directly examine the above question in plasma systems because factor VIIa added to factor VII–deficient plasma at a 10% of normal factor VII plasma concentration failed, in itself, to activate significant amounts of factor IX (Fig 6). When factor VII–deficient plasma was supplemented with a normal plasma concentration of either factor VIIa or factor VII, no difference in the activation of either factor IX or factor X was demonstrated (Fig 7). Overall, therefore, our data do not support the hypothesis that competition of factor VII with factor VIIa for limited tissue factor binding sites represents a control mechanism of physiological significance for coagulation during hemostasis. It leaves unexplained the observation of paradoxical instances of venous thrombotic disease in patients with hereditary factor VII deficiency.[1,22] Rather, a comparison of the activation curves for purified and plasma systems (see Fig 6) illustrates that a plasma material, the properties of which have been described earlier from this laboratory,[16] may function as a key regulator of tissue factor–induced coagulation.[16]

Our data support the concept, first proposed by investigators from Nemerson's laboratory from observations in purified systems,[18,23] that activation of factor VII by factor Xa is a key step in triggering tissue factor–dependent coagulation. The data herein provide further evidence (eg, compare the activation curves for purified and plasma systems of Fig 6) that a plasma material described earlier from this laboratory[16] and whose effectiveness also depends upon generation of factor Xa, may function as a key control mechanism for tissue factor–induced coagulation.[16]

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Deficiency of Platelet Membrane Glycoprotein Ia Associated With a Decreased Platelet Adhesion to Subendothelium: A Defect in Platelet Spreading

By H. Karel Nieuwenhuis, Kjell S. Sakariassen, Wim P.M. Houdijk, Patricia F.E.M. Nievelstein, and Jan J. Sixma

A bleeding disorder with absent collagen-induced platelet aggregation and adhesion has been described in a patient whose platelets failed to express surface glycoprotein Ia. We studied the interaction of her platelets with subendothelium in an annular perfusion chamber and the interaction with purified human collagen type III in a rectangular perfusion system under flow conditions. Platelet adherence was almost completely absent both at low and high shear rates. The few platelets which adhered remained in the contact stage without subsequent spreading and aggregate formation. Addition of a monoclonal antibody, which was directed against the von Willebrand moiety of FVIII-VWF, to the blood, completely abolished platelet adherence at high shear rates and had a partial effect at low shear rates. These data indicate that von Willebrand factor plays a role in the initial attachment (contact stage) of platelets to subendothelium. We conclude that the bleeding disorder and excessively prolonged bleeding time in our patient are caused by a new specific defect of the platelet-vessel wall interaction.

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FOLLOWING damage of the vessel wall blood platelets adhere to the exposed subendothelium. This process is an early and critical event in the formation of a hemostatic plug or thrombus. The perfusion system of Baumgartner,[1,2] in which deendothelialized artery segments are exposed to flowing blood, is a widely used model for the study of platelet adherence in vitro. By means of morphometrical evaluation two stages of platelet adhesion may be distinguished: (1) an initial attachment or contact phase and (2) the subsequent spreading of blood platelets on the surface.

Impaired platelet adherence has been demonstrated in patients with Bernard-Soulier Syndrome,[3-8] whose platelets are deficient in GPIb, and in patients with von Willebrand's disease, who have a deficiency or abnormality of factor VIII-von Willebrand factor (FVIII-VWF).[6,8-11] Perfusion studies in patients with von Willebrand's disease and studies with antibodies directed against von Willebrand Factor (VWF)[12,13] have demonstrated that both plasma von Willebrand factor and von Willebrand factor present in the vessel wall contribute to platelet adherence, but their effect is only observed at relatively high wall shear rates. Controversy exists, whether the decreased platelet adherence in von Willebrand's disease is due to impaired initial platelet attachment or to reduced platelet spreading.[14,15]

Recently we reported a patient with a new specific defect of platelet function. Her platelets were totally unresponsive to collagen and aggregated normally upon stimulation with other agonists.[16] The abnormality was associated with a deficiency of platelet membrane glycoprotein Ia. This report characterizes the abnormality of platelet adherence in this patient.

MATERIALS AND METHODS

Patient and normal subjects. Patient G, a 34-year-old woman, is a previously reported patient.[16] She had a history of easy bruising following minor trauma from her teens on. At the age of 21 she had an accident and a large subcutaneous hematoma developed from the groin to the ankle. Her menstrual bleedings have been heavy, but have been well-controlled with oral contraceptives and tranexamic acid (AMCA). There was no prolonged hemorrhage from small cuts; nose bleeding or spontaneous gingival bleeding did not occur so far. She underwent tonsilectomy (age 9) and appendectomy (age 15) without bleeding complications. A cesarian section (age 23) and tooth extractions (age 26 and 28) were performed without undue blood loss after cryoprecipitate infusion. A prolonged bleeding time and absent collagen-induced platelet aggregation were first discovered in 1973. Coagulation studies were normal and von Willebrand's disease was ruled out by repeated demonstrations of normal factor VIII-VWF parameters (VIII:C 140%, VWF:Ag 89%, VWF:ristocetin cofactor 94%, normal mobility of VWF in crossed immunoelectrophoresis). Her mother also showed easy bruising, but had not undergone any operative procedures or injuries. Her maternal grandfather had been admitted to the hospital several times because of severe epistaxis. Apart from this the family history was negative.

We recently reported[16] that the patient's platelets were totally unresponsive to collagen. High doses of several types of collagen induced neither shape change, secretion, nor aggregation. In contrast, her platelets aggregated normally in response to ADP, arachidonic acid, thrombin, PAF-acether, Ionophore A23187, epinephrine, and ristocetin. Study of platelet adherence to collagen in a static system according to Legrand[17] disclosed a severe defect of adhesion. Her platelets failed to express surface glycoprotein Ia (15% to 25% of normal).

Healthy adult volunteers from the hospital staff served as normal controls. The patient and the control subjects had not ingested any drugs for at least 2 weeks prior to the study. Informed consent was obtained from all donors and experiments were performed in accordance with the Declaration of Helsinki.

Blood collection. Blood samples were collected by venepunctures into polystyrene tubes containing 0.1 vol 110 mmol/L trisodium citrate.

Platelet interaction with subendothelium. The interaction of blood platelets in flowing blood with subendothelium was studied under steady flow[18] in an annular perfusion chamber according to Baumgartner.[1] Segments of a human umbilical artery were exposed for five minutes to flowing citrated blood at wall shear rates of 300 sec^{-1} and 1800 sec^{-1}. Before perfusion the endothelium was removed by brief exposure to air and the vessels were treated with aspirin in order to inhibit prostacyclin production.

Platelet interaction with subendothelium was quantified by a morphometric technique.[2] The artery segments were fixed in 2.5%

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glutaraldehyde after perfusion and subsequently postfixed in 2.0% osmium tetroxide. The specimens were embedded in epon, and sections of 1 μm thickness were stained at 70 °C for two minutes with basic fuchsin and methylene blue.[19] Platelet adherence was studied at a magnification of 1,000 × with an especially constructed micrometer in the ocular of the light microscope and evaluated as contact-platelets (platelets attached but not intimately bound to subendothelium) or spread-platelets (platelets at various stages of spreading on the subendothelium, including platelets at the base of platelet aggregates).

Platelet interaction with collagen. The interaction of blood platelets in flowing blood with purified human fibrillar collagen type III was studied with a rectangular perfusion chamber as previously described.[20,21] A glass microscope cover slip was coated with 30 μg/cm^2 of collagen type III by spraying with a retouching air brush. Collagen type III was isolated from human umbilical arteries as described.[20] Perfusion was performed during five minutes at wall shear rates of 300 sec^{-1} and 800 sec^{-1}. The platelet coverage was evaluated by light microscopy, with the help of a Quantimet 720 image analyzer (Imanco; Royston, UK).

Inhibition of FVIII-VWF–mediated platelet adherence with a monoclonal antibody against VWF. In some experiments perfusates were incubated and vessel segments were pretreated with CLB-RAg 35 as previously described.[13] Perfusates were incubated for ten minutes at 37 °C with CLB-RAg 35 (10 μL ascites/mL blood) before a perfusion run. CLB-RAg 35 (kindly provided by Dr J.A. van Mourik, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) is a monoclonal antibody against von Willebrand factor that inhibits ristocetin-induced platelet aggregation and VWF binding to platelets and that blocks VWF-mediated platelet adherence to subendothelium without interfering with the binding of VWF to collagen.[22,23]

Influence of an antibody against fibronectin on platelet adherence. In some experiments, vessel segments were pretreated with rabbit antihuman fibronectin F(ab')$_2$ fragments (Cappel Laboratories, Cochranville, Pa), as described,[24] and perfusion studies were performed using washed patient's platelets resuspended in fibronectin-free plasma[20] or in normal plasma. The perfusate was reconstituted by adding washed red cells to the resuspended platelets (hematocrit 0.4; platelet count 190 × 10^9/L).

Statistical analysis. Statistical analysis was performed by means of Student's t test, and considered significant at $P < 0.05$.

RESULTS

Platelet adherence to collagen type III and subendothelium. Perfusion studies in a rectangular perfusion chamber, in which human collagen type III fibrils, coated on glass microscope coverslips, were exposed to flowing citrated blood, revealed an impaired adherence of the patient's platelets both at low ($P < 0.001$) and high ($P < 0.001$) wall shear rates (Table 1). On light microscopical evaluation, the patient's platelets were deposited as single platelets along the collagen fibrils in contrast to control platelets that were deposited as aggregates.

Addition of a monoclonal antibody against factor VIII-von Willebrand Factor (CLB-RAg 35) to the blood caused a marked further reduction of the platelet deposition at high shear rate in the patient ($P < 0.001$) and the controls ($P < 0.001$). At low shear conditions, the antibody induced a decreased platelet deposition in the patient ($P < 0.001$) but not in the controls.

Platelet adherence to artery subendothelium when using the patient's blood was also severely decreased (Table 1). Morphometric evaluation disclosed that the resultant platelet adhesion consisted of platelets in the contact stage, but not in the spread stage (Fig 1). Platelet aggregates were absent. In contrast, when using blood of normal subjects, platelet adhesion was predominantly composed of spread platelets and about 30% to 40% of the spread platelets were covered with aggregated platelets.

Addition of CLB-RAg 35 to the blood together with preincubation of the vessel wall with the antibody (in order to block VWF present in the subendothelium) completely abolished contact of the patient's platelets with the subendothelium at high shear rate ($P < 0.001$), but had only a slight nonsignificant effect at low shear rate. In the controls, the antibody totally inhibited platelet adhesion at high shear rate ($P < 0.001$) and reduced adhesion to a less but significant extent ($P < 0.01$) at low shear rate.

Preincubation of the vessel wall with antifibronectin and perfusion with patient's platelets resuspended in fibronectin-free plasma gave a similar low platelet deposition at low shear rate as perfusion with normal plasma without preincubation of the vessel wall.

DISCUSSION

In a previous report we described a patient with easy bruising and a prolonged bleeding time, whose platelets were totally unresponsive to collagen; other agonists, however,

Table 1. Platelet Adherence to Purified Fibrillar Collagen Type III and Human Artery Subendothelium

	Platelet-Collagen Interaction		Platelet-Subendothelium Interaction			
	Wall Shear Rate (sec^{-1})	Surface Coverage (%)	Wall Shear Rate (sec^{-1})	Total Surface Coverage (%)	Contact (%)	Spread (%)
Control	300	4.2 ± 0.5 (4)	300	22.2 ± 1.2 (5)	2.2 ± 0.6	20.0 ± 1.5
	800	10.5 ± 0.9 (4)	1800	43.0 ± 2.3 (5)	3.4 ± 0.4	39.6 ± 1.9
Patient	300	1.5 ± 0.2 (5)	300	5.3 ± 0.9 (8)	5.3 ± 0.9	0.0 ± 0.0
	800	2.3 ± 0.3 (5)	1800	5.0 ± 0.4 (8)	5.0 ± 0.4	0.0 ± 0.0
Control + CLB RAg 35	300	4.0 ± 0.6 (5)	300	9.5 (2)	1.0	8.5
	800	0.7 ± 0.2 (5)	1800	1.0 (2)	0.0	1.0
Patient + CLB RAg 35	300	0.2 ± 0.0 (5)	300	4.0 ± 1.3 (5)	4.0 ± 1.3	0.0 ± 0.0
	800	0.0 ± 0.0 (5)	1800	0.7 ± 0.4 (5)	0.7 ± 0.4	0.0 ± 0.0
Patient + fibronectin*			300	1.7 ± 0.6 (3)	1.7 ± 0.6	0.0 ± 0.0
Patient – fibronectin†			300	1.0 ± 0.0 (3)	1.0 ± 0.0	0.0 ± 0.0

Data are given as mean ($n = 2$) or mean ± SEM ($n \geq 3$) with the number of perfusions (n) in parentheses. The control values are results from studies with five different normal subjects.

*Patient's platelets mixed with normal plasma and erythrocytes, without preincubation of the vessel wall.

†Patient's platelets mixed with fibronectin-free plasma and erythrocytes, with preincubation of the vessel wall with antifibronectin.

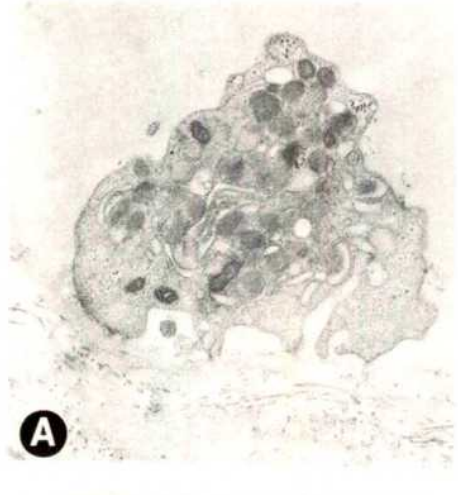

Fig 1. Platelet interaction with subendothelium of a human umbilical artery. The artery segments were fixed and embedded following five minutes of perfusion with blood. The platelets of the patient adhere as single platelets, do not spread out on the subendothelium, and still contain their granules: (A) electron and (B) light microscopical picture. Platelets of a control (C) are spread out on subendothelium and form small aggregates.

induced normal aggregation.[16] Von Willebrand's disease was ruled out, and two-dimensional electrophoretic analysis of radioiodinated platelet membrane glycoproteins showed a deficiency of the major platelet plasma membrane glycoprotein GPIa, suggesting that this glycoprotein could be involved in the platelet-collagen interaction. Investigation of the interaction of her platelets with collagen in a static system with a technique based upon gel filtration of nonadhesive platelets through a Sepharose 2B column after incubation of platelets with collagen disclosed a severe defect of adhesion.

In this investigation we studied the adhesion of her platelets to subendothelium and purified human collagen type III under flow conditions similar to those found in vivo, and evaluated platelet adherence with a morphometric technique in order to distinguish the initial attachment (contact) and subsequent spreading.

The adherence of our patient's platelets to collagen and subendothelium was severely impaired and was characterized by a total absence of spread platelets on subendothelium. This defect differs completely from the adhesion defects in von Willebrand's disease and Bernard Soulier syndrome. In contrast to these disorders, the patient's platelets were unable to spread on exposed subendothelium both at low and high shear rates and, apparently as a result of this, aggregates were absent.

The few platelets of our patient that adhered to the subendothelium were bound to the vessel wall by a small area of the membrane. This contact could be an interaction of collagen, von Willebrand factor, and the platelet membrane receptor for VWF, or an interaction of collagen with the binding site of the collagen receptor. In order to define the role of VWF in the contact stage, we performed perfusion studies with a monoclonal antibody against VWF. VWF completely mediated contact in the patient at high wall shear rates (comparable with those in the microvasculature) and had a partial effect at low shear conditions. It is not clear as yet which factor is responsible for the remaining contact at low shear rate. In this and a previous study[24] we could not demonstrate a role for fibronectin at low shear rate.

These data support the hypothesis that the defect in platelet adhesion in von Willebrand's disease is associated with a reduced ability of platelets to attach to subendothelium upon initial contact.[15] The attachment of the patient's collagen-unresponsive platelets to subendothelium via VWF at high shear rate indicates that platelets can adhere via VWF without any stimulation by collagen. This agrees with studies[11,14] that showed that VWF first binds to subendothelium and then mediates platelet adherence, suggesting that VWF undergoes a structural alteration on subendothelium that enables it to bind to a platelet receptor. Stimulation of platelets subsequently can expose other binding sites (like GPIIb-III) for VWF,[25-27] but its physiologic significance is not clear as yet because of the inhibition of this binding by fibrinogen.[28]

The absolute failure of the patient's platelets to spread on exposed subendothelial matrix and purified collagen points to the important role of collagen in platelet activation, subsequent spreading, and aggregate formation. We conclude that the hemorrhagic diathesis and the prolonged bleeding time in our patient can be ascribed to a new, specific defect of the platelet-vessel wall interaction. The mildness of the bleeding disorder, however, points to the important role of other agonists, such as thrombin, in hemostasis.

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Immunohistochemical Localization of Membrane and α-Granule Proteins in Plastic-Embedded Mouse Bone Marrow Megakaryocytes and Murine Megakaryocyte Colonies

By Paula E. Stenberg, Jay H. Beckstead, Rodger P. McEver, and Jack Levin

Using an immunoperoxidase technique that permits optimal antigen localization at the light microscope level, we have detected two platelet α-granule constituents and three platelet membrane glycoproteins in mouse bone marrow megakaryocytes and in murine megakaryocyte colonies grown in soft agar culture for three to seven days. Using polyclonal antibodies prepared against human platelet proteins, we have demonstrated labeling for von Willebrand factor, fibrinogen, and the membrane glycoproteins IIIa and GMP-140 in both bone marrow megakaryocytes and megakaryocyte colonies after seven days of culture. Using monoclonal antibodies to membrane glycoproteins IIb and GMP-140, we have demonstrated label in mouse bone marrow megakaryocytes. Granulocyte and macrophage colonies were negative for each of these markers. Murine bone marrow megakaryocytes and megakaryocyte colonies demonstrated a similar enzyme histochemical pattern: weakly positive for α-naphthyl acetate esterase and negative for chloroacetate esterase. These data indicate that megakaryocytes grown in soft agar culture express many of the same glycoproteins as bone marrow megakaryocytes. Furthermore, the ability of antibodies directed against human platelet membrane glycoproteins to identify murine megakaryocyte glycoproteins indicates that these constituents have been highly conserved during evolution.

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THE development of animal models and marrow culture systems has improved our understanding of megakaryocytopoiesis, but morphologic recognition of the earliest cells of the megakaryocyte lineage has been hampered by the lack of specific markers. Acetylcholinesterase, an enzyme restricted to megakaryocytes in rodent bone marrow, has been extensively employed, but additional markers would be advantageous. Recent studies of human megakaryocytes have demonstrated a number of useful markers. A peroxidase specific for the megakaryocyte-platelet lineage, localized to the endoplasmic reticulum and perinuclear envelope of the megakaryocyte and the dense tubular system of the platelet, has been demonstrated ultrastructurally.[1] More recently, antibodies specific for platelet membrane glycoproteins have been employed for identification of human megakaryocytes in vivo and in vitro.[2-6] However, reports of morphologic studies of megakaryocytes in tissue culture systems have been limited thus far.[3,7-12]

Using immunohistochemical techniques on plastic-embedded sections[13] and antibodies to human platelet constituents, we have detected plasma membrane glycoproteins IIIa and IIb, an alpha granule membrane glycoprotein (GMP-140), and alpha granule glycoproteins von Willebrand factor and fibrinogen in both murine bone marrow megakaryocytes and megakaryocyte colonies grown in soft agar cultures. This strongly suggests that currently available, well-characterized antibodies against antigens of the human megakaryocyte-platelet lineage can be utilized to identify and study murine megakaryocytes and their precursors.

MATERIALS AND METHODS

Animals

C57BL/6J mice, obtained from Simonsen Laboratories, Inc (Gilroy, Calif), were used for these experiments. These mice were bred under specific pathogen-free conditions and then maintained under conventional conditions. Guinea pigs and rats were purchased from EZH Co (Williams, Calif).

Preparation of Murine, Guinea Pig, and Rat Bone Marrow Specimens

Mouse femoral marrow samples were obtained from animals killed by cervical dislocation and guinea pig and rat femoral marrow samples were obtained from animals killed by ether anesthesia. The femur was split lengthwise and the fragments of marrow gently pushed out from the bone into 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, at 4 °C. Specimens of marrow were fixed for four to six hours at 4 °C.

Preparation of Murine Colony Samples

Spleen cells were obtained as previously described.[14] All cultures were performed in an agar medium (final concentration of agar was 0.3% in Dulbecco's modified Eagle's medium).[14] Final concentration of horse serum was 20%. One mL of a cell suspension in agar medium was cultured with 0.1 mL of pokeweed mitogen stimulated spleen cell–conditioned medium, and incubated for three to seven days in a humidified atmosphere of 10% CO_2 in air. At various times, colonies were either removed from culture dishes with a finely drawn Pasteur pipette and placed in the fixative solution, or fixative was gently poured over the entire agar disc. In the latter case, the discs then were gently removed from the 35 mm plastic dishes and placed in a larger volume of fixative. All samples were fixed at 4 °C in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for four to six hours.

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Preparation of Bone Marrow and Colony Samples for Plastic Embedding

The following steps were performed at 4 °C: Colonies in suspension were processed by allowing them to settle by gravity after each step. After fixation, samples were rinsed three times with 0.1 mol/L phosphate buffer which contained 2% sucrose and 50 mM NH_4Cl. The samples were then dehydrated in 50% acetone for 0.5 hour, 95% acetone for 0.5 hour, 100% acetone for 0.5 hour, and 50 parts acetone/50 parts glycol methacrylate monomer for 0.5 hour followed by infiltration with 100% glycol methacrylate monomer overnight. Subsequently, the specimens were embedded in a mixture composed of glycol methacrylate monomer (20 mL), benzol peroxide (0.09 g), and polyethylene glycol 400 with N,N-dimethylaniline (0.5 mL; Polysciences Inc, Warrington, Pa). The embedded tissue was placed under vacuum (15 to 20 mm Hg) at 4 °C and the plastic allowed to polymerize overnight. One to three um sections were then cut with glass knives on a Sorvall JB-4 microtome. The sections were transferred via water to coverslips and air dried at room temperature.

Enzyme Histochemical and Immunohistochemical Procedures

Routine staining procedures and enzyme histochemistry were performed as previously described.[15,16] The immunohistochemical procedure[13] is a modification of a biotin-avidin procedure.[17] Immunohistochemical procedures on plastic sections were performed in the following sequence. Unless otherwise specified, all incubations were carried out at room temperature.

(*a*) Digestion with 0.25% trypsin in Saline A (NaCl, 150 mmol; KCl, 5 mmol; glucose, 5 mmol; phenol red, 0.01 mmol; $NaHCO_3$, 7 mmol) (Gibco, Santa Clara, Calif), pH 7.6, for ten minutes at 37 °C.

(*b*) Incubation with 3% normal goat or horse serum (same species as secondary antibody) in Dulbecco's Ca++- and Mg++-free phosphate-buffered saline (CMF-PBS) (KCl, 3 mmol; KH_2PO_4, 1 mmol; NaCl, 150 mmol; $Na_2HPO_4.7H_2O$, 8 mmol; pH 7.36 to 7.45) for 30 minutes at 37 °C.

(*c*) Incubation overnight at 4 °C with one of the antisera listed below. All dilutions were made in CMF-PBS with 3% normal goat or horse serum (same species as secondary antibody, vide infra) and sections were rinsed between incubations with CMF-PBS.

(1) Polyclonal antibodies: rabbit antihuman von Willebrand factor (Factor VIII-related antigen) (Dako, Santa Barbara, Calif, 1:1000); rabbit antihuman fibrinogen antiserum (Cappel, Malvern, Pa, 1:1000); rabbit antihuman platelet membrane glycoprotein IIIa[18] (1:2000); rabbit antihuman α-granule membrane glycoprotein (GMP-140)[19] (1:2000); rabbit antimouse platelet antiserum (1:500)[20]; rabbit antirat brain acetylcholinesterase (Dr Jean Massoulie, Paris, France, 1:100); rabbit antihuman lysozyme (Dako, Santa Barbara, Calif, 1:500).

(2) Monoclonal antibodies: mouse antihuman platelet GMP-140 (S12)[21](1:500); mouse antihuman glycoprotein IIb (Tab)[22] (1:500).

(*d*) Incubation with the appropriate secondary antibody, ie, biotinylated goat antirabbit IgG (Vector, Burlingame, Calif, 1:200) or biotinylated horse antimouse IgG (Vector, 1:200) for one hour.

(*e*) Inhibition of endogenous peroxidase by incubation with 0.3% H_2O_2 in 30% methanol for three minutes.

(*f*) Incubation with the avidin-biotin-peroxidase complex (Vector, 1:80) for one hour.

(*g*) Development of peroxidase reaction by preincubation with 0.05% 3,3-diaminobenzidine in CMF-PBS for ten minutes, followed by incubation with a CMF-PBS solution containing 0.05% diaminobenzidine, 0.1% H_2O_2, 0.1 mol/L imidazole, and 0.1 mol/L sodium azide for five minutes. After washing 3 times in water, the reaction was further enhanced by incubation with a solution of 0.5% $CuSO_4$ for five minutes.

(*h*) Counterstaining with Gill's hematoxylin #2 for 1.5 minutes; blueing with Scott's water (0.2% Na_2CO_3, 1% $MgSO_4$ in tap water); and finally drying and mounting with Permount.

Control procedures included the substitution of irrelevant antibodies, preimmune serum, or ascites fluid for the primary antibodies and the elimination of primary and secondary reagents.

RESULTS

We have utilized polyclonal and monoclonal antibodies directed against a variety of human platelet proteins to localize these substances within the megakaryocytes of intact specimens of rodent bone marrow and murine megakaryocytes obtained from soft agar cultures. These proteins can be classified, by their fine-structural localization in platelets, into three groups: (1) plasma membrane proteins, (2) α-granule membrane proteins, or (3) α-granule matrix proteins. Since these antibodies against human platelet proteins strongly cross-reacted with rodent antigens, we were able to investigate their localization in bone marrow cells and in cells at early culture times. Control preparations showed no evidence of nonspecific staining.

Staining With Antibodies to Platelet Plasma Membrane Proteins

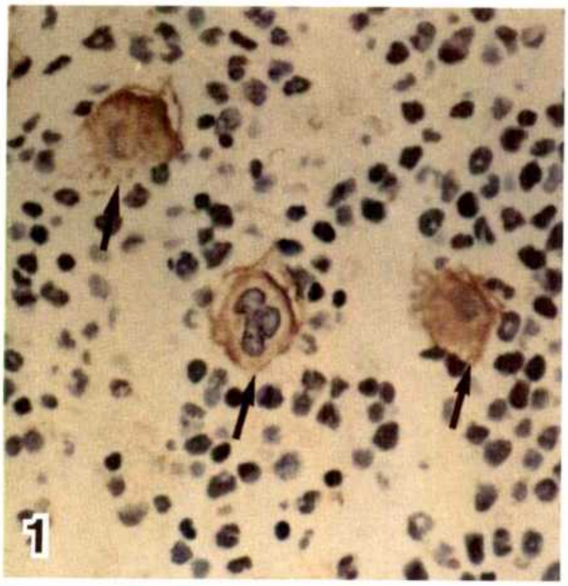
Glycoprotein IIIa. Polyclonal antisera against human glycoprotein IIIa labeled virtually all morphologically identifiable murine bone marrow megakaryocytes (Fig 1). Staining in megakaryocytes was distributed throughout the cytoplasm but was most intense on the plasma membrane. No staining of other hematopoietic cells was observed. Guinea pig and rat megakaryocytes from bone marrow specimens were also labeled (not shown), but not as strongly as murine megakaryocytes.

Murine megakaryocyte colonies, fixed and processed three days after initiation of soft agar cultures, were strongly reactive for GP IIIa (Fig 2), as were colonies processed seven days after culture (Fig 3). Granulocyte colonies (Fig 4) and macrophage colonies (Fig 4, inset) from the same preparations were completely unreactive for GP IIIa.

Glycoprotein IIb. Monoclonal antibodies against human glycoprotein IIb (Tab) labeled virtually all morphologically identifiable murine bone marrow megakaryocytes (not shown). The staining pattern was similar to that obtained with antibodies to GP IIIa (vide supra).

Platelet Antiserum. Heterologous antiserum produced in rabbits against mouse platelets (PAS) labeled virtually all morphologically identifiable murine bone marrow megakaryocytes (not shown). The staining pattern was similar to that obtained with antibodies to GP IIIa (vide supra).

Murine megakaryocyte colonies, fixed and processed five to seven days after initiation of soft agar cultures, were strongly labeled with PAS (not shown). Granulocyte and macrophage colonies from the same preparation were unlabeled with PAS (not shown).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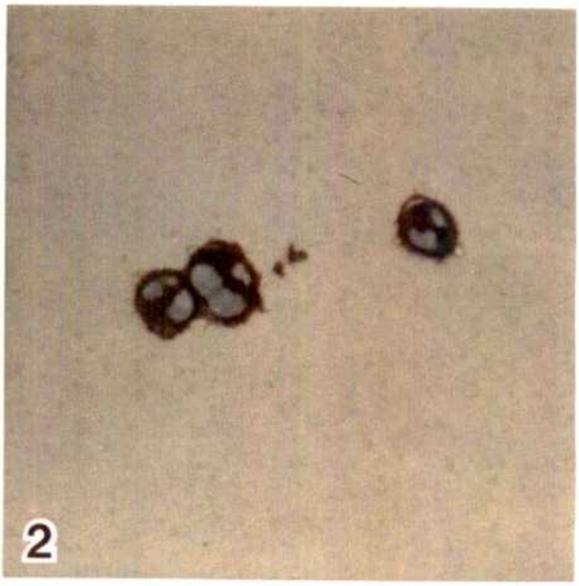
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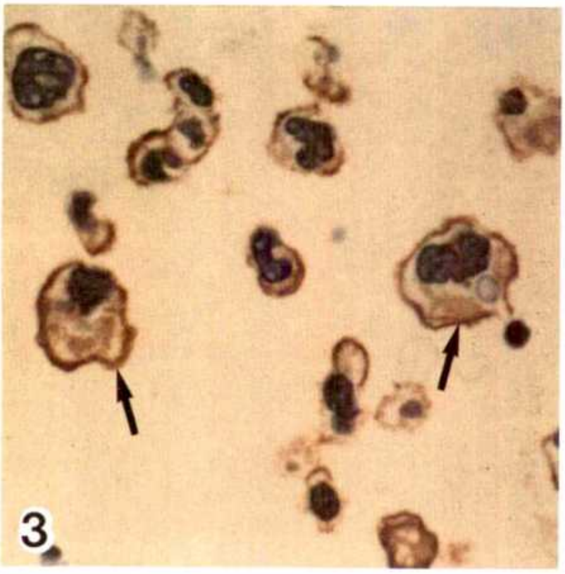
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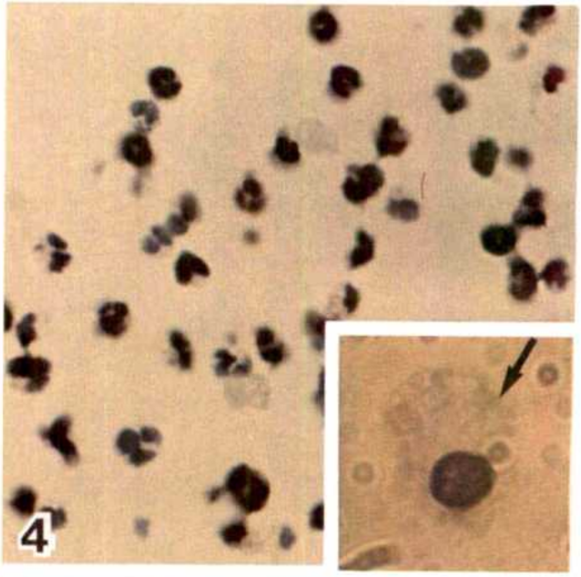
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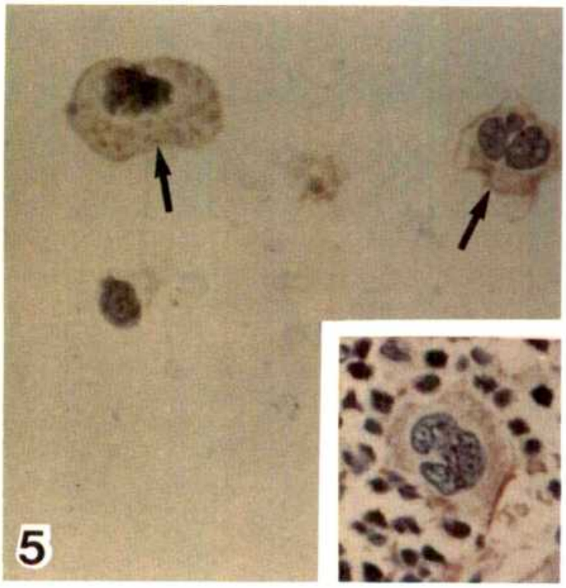
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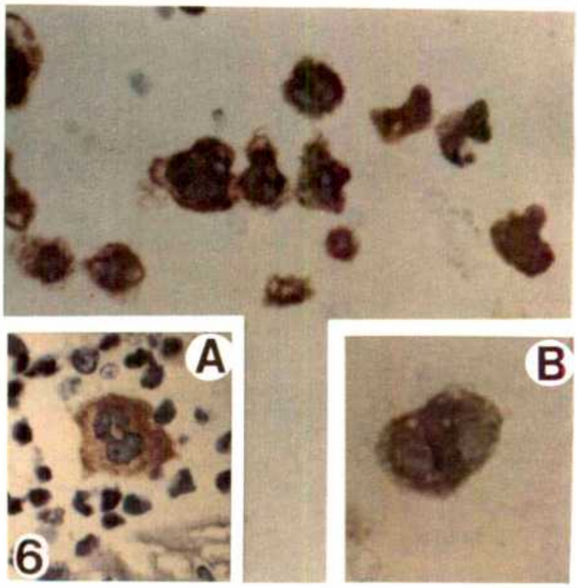
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Staining with Antibodies to Platelet α-Granule Membrane Protein 140

Both polyclonal (not shown) and monoclonal (S12) (Fig 5, inset) antibodies developed against the human platelet α-granule membrane protein with a molecular weight of 140 kD (GMP-140) produced labeling of virtually all morphologically identifiable murine bone marrow megakaryocytes. The staining pattern was similar to that observed with the antibodies against platelet α-granule matrix proteins (vide infra) and is consistent with the fine-structural localization of GMP-140 in human platelets.[19] Labeling with the monoclonal antibody S12 was weaker than the labeling obtained with the polyclonal antibodies. All other hematopoietic cells were negative.

Polyclonal antibodies produced variable labeling of megakaryocyte colonies processed seven days after initiation of cultures of splenic cells (Fig 5). Some cells exhibited a strong granular label while others, usually smaller in size, appeared negative. The monoclonal antibody S12 produced only weak labeling of megakaryocytes from the same preparation (not shown). Granulocyte and macrophage colonies were negative (not shown).

Staining with Antibodies to Platelet α-Granule Matrix Proteins

Von Willebrand Factor. Antibodies against human von Willebrand factor (VWF) produced strong granular staining in virtually all morphologically identifiable murine bone marrow megakaryocytes (Fig 6, inset A). The distribution was similar to that observed with antibodies to GMP-140. There was no labeling of other hematopoietic cells, but moderate endothelial cell staining was observed (not shown).

Murine megakaryocyte colonies, processed three days (Fig 6, inset B) to seven days (Fig 6) after initiation of cultures, were strongly reactive with antibodies to VWF. Granulocyte and macrophage colonies were negative.

Fibrinogen. Antibodies against human fibrinogen produced a strong granular label in virtually all morphologically identifiable murine bone marrow megakaryocytes (not shown). No other hematopoietic cells were labeled.

Murine megakaryocyte colonies processed five to seven days after initiation of cultures were strongly reactive for fibrinogen (not shown). Granulocyte and macrophage colonies were negative.

Controls

When preimmune rabbit serum was substituted for specific primary polyclonal antiserum, no staining was observed in any cells. Similarly, when either primary or secondary reagents were eliminated, no staining was observed. When ascites fluid was substituted for specific monoclonal antibodies, no staining was observed in any cells.

Other Markers

Polyclonal antisera produced in rabbits against rat brain acetylcholinesterase labeled many, but not all, morphologically identifiable murine bone marrow megakaryocytes (not shown). Polyclonal antisera produced in rabbits against human lysozyme labeled most murine bone marrow granulocytes and macrophages and cells in granulocyte and macrophage colonies, but not bone marrow megakaryocytes or megakaryocytes grown in soft agar culture (not shown).

Both murine bone marrow megakaryocytes and megakaryocyte colonies obtained from seven-day cultures of splenic cells were positive for the enzyme α-naphthyl acetate

Specimens of normal murine bone marrow and samples of murine megakaryocyte colonies were embedded in plastic and tested immunohistochemically for a variety of platelet antigens. The brown peroxidase reaction product is present at sites of antigen localization. The cells were stained with hematoxylin.

Fig 1. Bone marrow megakaryocytes (arrows) labeled with polyclonal antibodies to human GP IIIa demonstrate brown staining which is easily identified at this magnification. Other cells in this field are negative (original magnification × 600).

Fig 2. Megakaryocytes from a big cell colony type (Levin et al, 1981) processed three days after initiation of cultures of splenic cells in soft agar and stained with polyclonal antibodies to GP IIIa. The cells are strongly reactive (original magnification × 600).

Fig 3. Megakaryocytes from a heterogeneous colony type, processed seven days after initiation of cultures, and stained with polyclonal antibodies to GP IIIa. The megakaryocyte plasma membranes (arrows) are heavily labeled, while the cytoplasm of these cells shows a lighter, diffuse reaction. The membranes are heavily stained even in smaller, more immature megakaryocytes (original magnification × 600).

Fig 4. Granulocyte colony and macrophage from a representative colony (inset) processed after seven days of culture and stained for the presence of GP IIIa. The cytoplasm and plasma membranes of both granulocytes and macrophage are completely negative. Arrow indicates plasma membrane of macrophage (inset) (original magnification × 900; inset × 900).

Fig 5. Megakaryocytes from a heterogeneous colony type, processed seven days after initiation of cultures, and stained with polyclonal antibodies to GMP-140, an alpha-granule membrane protein. The two large cells in this field demonstrate label in a granular pattern within the cytoplasm (arrows) while a third, smaller cell is unreactive. Inset: A megakaryocyte from a bone marrow specimen, labeled with the monoclonal antibody S12 which recognizes the same 140 kD glycoprotein as the polyclonal anti-GMP-140 antibody. The cell cytoplasm is positive (original magnification × 600; inset × 600).

Fig 6. Megakaryocytes from a heterogeneous colony type, processed seven days after initiation of cultures, and stained with polyclonal antibodies to VWF. Most of the cells in this field are labeled. Inset A: A megakaryocyte from a bone marrow specimen, positively stained for VWF. Inset B: A megakaryocyte from a big cell colony type, processed three days after initiation of cultures, positively stained for VWF (original magnification × 600; inset A × 600; inset B × 900).

esterase, but more weakly positive than human megakaryocytes (not shown). Some variability in the degree of staining was noted within the same bone marrow specimen. Granulocyte and macrophage colonies were negative. Murine bone marrow megakaryocytes and macrophages and megakaryocyte and macrophage colonies obtained after seven days of culture were negative for the enzyme chloroacetate esterase (not shown). In contrast, neutrophils in both bone marrow and granulocyte colony samples were strongly reactive for this enzyme (not shown).

DISCUSSION

Utilizing monoclonal and polyclonal antihuman antibodies and an immunoperoxidase technique, we have demonstrated the presence of a variety of platelet membrane and alpha granule glycoproteins in murine bone marrow megakaryocytes and in murine megakaryocytes obtained from colonies grown in soft agar cultures. In an earlier report[23] we demonstrated the presence of these glycoproteins in human bone marrow megakaryocytes using the same antibodies and immunohistochemical technique. All antihuman megakaryocyte/platelet antibodies strongly cross-reacted with murine megakaryocytes. In contrast, antihuman lysozyme antibodies labeled murine granulocytes and macrophages, but not megakaryocytes. Guinea pig and rat bone marrow megakaryocytes were also reactive with antibodies to glycoprotein IIIa (the only antibody tested against these species). Therefore, we have concluded that these glycoproteins are highly conserved. Support for this conclusion has come from a recent study by Kunicki and Newman[24] in which they demonstrated that human platelet membrane glycoproteins IIb and IIIa are structurally conserved in chicken peripheral blood thrombocytes. They showed this by indirect immunofluorescence, using polyclonal and monoclonal antibodies, and two-dimensional polyacrylamide gel electrophoresis, which revealed the incorporation of ^{35}S methionine by chicken thrombocytes into glycoprotein IIb and IIIa analogs in vitro. Unexpectedly, in our study, mouse bone marrow megakaryocytes also labeled with monoclonal antibodies to human platelet membrane glycoproteins, although not as strongly as with polyclonal antibodies. Since the monoclonal antibodies were produced in Balb C mice and were used to examine cells from C57BL/J6 mice, this suggests that there may be some differences between platelet glycoproteins of these species.

At least two types of murine megakaryocyte colonies have been recognized in soft agar cultures.[20,25–30] One type is typically composed of 10 to 30 large megakaryocytes which are mature in appearance ("big cell" type). The other ("heterogeneous" type) is composed of more cells (often greater than 100), some of which are small and immature as evaluated in part by staining for acetylcholinesterase, a specific marker for murine megakaryocytes.[26] In our current studies, we observed an increase in average cell size from 3 to 7 days in culture, and the size of megakaryocytes in seven-day colonies (at which time murine megakaryocyte colonies have reached their maximal maturation in our culture system) approximated the size of mature bone marrow megakaryocytes. However, no evidence of platelet production was observed.

Since both colony types appear simultaneously in agar cultures, they probably represent the progeny of different populations of precursors.[26,29] Our data demonstrate that although each colony type probably originates from different populations of precursors, the appearance of markers, as detected by light microscope level immunohistochemical analysis, is similar in both, since after seven days of culture, the majority of cells in both "big cell" and "heterogeneous" colonies labeled for all proteins tested. We also demonstrated that murine megakaryocyte colonies, present after only three days of soft agar cultures of murine colony-forming cells, labeled for both platelet membrane glycoprotein IIIa and von Willebrand factor, an alpha granule matrix protein. This is significant since cells at this time in culture are morphologically immature; in fact, this is the first day of culture in which murine megakaryocyte colonies may be reliably visually identified, based upon the relatively large size and refractility of the cells.

Previously, staining for acetylcholinesterase has been used to identify immature megakaryocytes in the mouse.[31–33] Megakaryoblasts have been identified in human bone marrow by platelet peroxidase, the earliest marker of megakaryocytes,[1,2] and by antisera against platelet membrane preparations and platelet membrane glycoproteins.[5,34] Furthermore, Vainchenker et al[7] identified human megakaryocytes in plasma clot cultures by the presence of platelet peroxidase, and Mazur et al[3] identified human megakaryocyte colonies with immunofluorescence techniques using an antiplatelet glycoprotein antiserum. Significantly, Vainchenker et al[2] and Vinci et al,[35] using monoclonal antibodies, determined that the glycoprotein IIb/IIIa complex is an early antigenic marker of megakaryocyte maturation, detectable in cells at five or six days after initiation of plasma clot cultures. However, our studies utilize for the first time, in soft agar cultures, antibodies to platelet membrane glycoproteins and other markers for identification of murine megakaryocytes at an early maturation point. The specificity of these markers is further confirmed by the presence of lysozyme in murine granulocytes and macrophages, but not in megakaryocytes. From our studies, we conclude that the presence of these various antigens in megakaryocytes, derived from colony-forming cells in vitro, is another indication that megakaryocytes and their precursors synthesize von Willebrand factor, fibrinogen, and membrane glycoproteins IIIa, IIb, and GMP-140.

Recently, it has been shown that the populations of megakaryocytes in different types of murine megakaryocyte colonies have different ploidy distributions.[20,26,28,29,36] The mean ploidy level of the "big cell"type was 16.8N/cell while the "heterogeneous" type had a mean ploidy level of only 6.8 N/cell; approximately half the cells in the latter type of colony contained 2N or 4N levels of DNA.[26] Our studies suggest that cells at low ploidy levels expressed platelet membrane and alpha granule glycoproteins, since almost all cells in both colony types were reactive for these markers by seven days or earlier in culture. Previous studies[37,38] have shown that megakaryocytes with ploidy levels ranging from 8N to 128N can achieve cytoplasmic maturity. Our data are consistent with Paulus' observation that organelle formation is initiated in immature megakaryocytes and reaches a peak after completion of DNA synthesis (ie, endomitosis).[37] It is unknown whether the pattern of protein expression may be altered by the maturational state of the megakaryocyte, as is the case, for example, in neutrophils, in which azurophil granules are formed only during the promyelocyte stage and specific granules are formed during the myelocyte stage.[39] Studies are therefore in progress to further correlate maturation and ploidy levels of the cells of megakaryocyte colonies with the pattern of appearance of these various glycoproteins.

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A Lower Molecular Weight γ-Chain Variant in a Congenital Abnormal Fibrinogen (Kyoto)

By Nobuhiko Yoshida, Minoru Okuma, Masaaki Moroi, and Michio Matsuda

A γ-chain variant with a lower molecular weight than the normal γ chain was detected in a new congenital abnormal fibrinogen with impaired polymerization of the fibrin monomer and with normal release of fibrinopeptides A and B in a 45-year-old male. Purified fibrinogen analyzed on SDS-polyacrylamide gel electrophoresis under the reduced condition contained an abnormal protein band with an apparent molecular weight of 48,000 compared with the γ chain with a molecular weight of 50,000. This abnormal protein band was found to be a γ-chain variant from the molar ratio of Aα chain : B β chain : γ chain : abnormal protein (about 2 : 2 : 1 : 1), with positive staining for carbohydrate and crosslinking ability. Crosslinked fibrin contained three types of γ-γ dimers with apparent molecular weights of 94,000 (the same as normal major γ-γ dimer), 92,000 and 90,000, and the plasmic digests of crosslinked fibrin in the presence of calcium retained three types of γ-γ dimer remnants. This suggests that the abnormal γ-chain variant has a shorter polypeptide chain not in the NH_2-terminal but in the COOH-terminal portion, probably at or near the polymerization site. This patient's two daughters had the same abnormal fibrinogen. This unique inherited abnormal fibrinogen was designated as fibrinogen Kyoto, and the γ-chain variant as γ_{Kyoto}.

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A LINEAR end-to-end association of fibrin monomer molecules is known to occur through the interaction of the thrombin-exposed polymerization site in the NH_2-terminal region of the fibrin monomer and the preexisting polymerization site in the COOH-terminal region of the γ chain, segment 374-396.[1-3] Among abnormal fibrinogens with impaired polymerization of fibrin monomers, those with defective γ chains are very rare[4-8] (fibrinogens Paris I,[4] Nagoya,[5] Bern I,[6] Haifa,[7] and Grenoble[8]) and γ-chain variants with an abnormal molecular weight are limited to Paris I[4] and Grenoble,[8] which have a larger molecular weight γ chain than normal. We describe here a new γ-chain variant, γ_{Kyoto}, with a lower molecular weight than normal, which was found in congenital dysfibrinogenemia, fibrinogen Kyoto, with defective polymerization of the fibrin monomer.

MATERIALS AND METHODS

Blood collections and coagulation studies. Venous blood was collected in one-tenth volume of 3.8% sodium citrate or in acid citrate dextrose (ACD). Platelet-rich plasma and platelet-poor plasma were prepared for the platelet aggregation and blood coagulation studies, respectively, as described previously.[9] Coagulation studies were performed according to procedures described elsewhere.[10-13] The reptilase time was done in a test system containing 0.1 mL plasma, 0.1 mL Tris-saline, pH 7.4 with or without 25 mmol/L $CaCl_2$, and 0.1 mL of 50 μg/mL enzyme (venom of Bothrops atrox, Pentapharm, Switzerland).

Purification of fibrinogen. Fifty mL of ACD plasma was mixed with 5 mmol/L benzamidine, 10 KIU/mL aprotinin, and 2 mmol/L EDTA, and passed through a column of lysine-Sepharose 4B (25 mL) and a column of gelatin-Sepharose 4B (25 mL) that were connected in tandem and equilibrated with 50 mmol/L Tris-HCl, 0.1 mol/L NaCl, 5 mmol/L benzamidine, 10 KIU/mL aprotinin, 2 mmol/L EDTA, pH 7.4, at room temperature, and columns were washed with the same buffer. Two hundred mL of unbound pass through fractions was pooled, brought to 25% saturation with saturated ammonium sulfate solution, stirred for 30 minutes, and centrifuged at 13,000 × g for 30 minutes. The pellet was washed two times with 25% ammonium sulfate solution, suspended in an appropriate amount of 0.3 mol/L NaCl, dialyzed extensively against the same solution at 4 °C, and finally the insoluble portion was removed by centrifugation at 13,000 × g for 30 minutes. The recovery of purification was 75% and thrombin clottability was 92%. Purified fibrinogen was stored at −80 °C until use.

Studies on purified fibrinogen. Fibrin monomer aggregation was studied according to the method of Belitser et al[14] as described by Gralnick et al.[15] Release of fibrinopeptides A and B was examined by high-performance liquid chromatography.[16] Release of fibrinopeptide A was also measured by radioimmunoassay.[17] Immunoelectrophoresis was done as previously described.[13] DEAE-Sephacel chromatography of fibrinogen was carried out by applying 10 mg of fibrinogen to a 40 mL column.[18] Fibrinmonomer-Sepharose chromatography of fibrinogen was carried out as described previously.[13] Crosslinked fibrin was prepared by adding 5 U/mL bovine thrombin and 1 U/mL purified human factor XIII[19] to fibrinogen (2 mg/mL) in 50 mmol/L Tris-HCl, 0.135 mol/L NaCl, 25 KIU/mL aprotinin, 5 mmol/L $CaCl_2$, pH 7.4. Reaction mixtures were incubated at 37 °C for 80 minutes, and the reaction was terminated by the addition of 2% SDS and 10% β-mercaptoethanol and incubation at 100 °C for 30 minutes. Plasmic digests of crosslinked fibrin in the presence of calcium were prepared essentially as described previously.[13] Fibrinogen (1 mg) was incubated with 2 U of bovine thrombin and 0.1 U of factor XIII in 1 mL of 50 mmol/L Tris-HCl, 0.135 mol/L NaCl, 10 mmol/L $CaCl_2$, pH 7.4, for two hours at 37 °C and for another 12 hours at 4 °C. Fibrin clots were squeezed out with a bamboo stick, washed 2 times with the above buffer, and digested by the addition of 0.25 mL of 0.013 mg/mL (0.4 CTAU/mL) human plasminogen[11] and 172 u/mL streptokinase in the above buffer (plasmin solution) for six hours at 37 °C. After six hours an additional 0.25 mL of plasmin solution was added, and the incubation was carried out for an additional 18 hours at 37 °C. The reaction

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was terminated by the addition of 500 KIU/mL aprotinin and 0.1 mmol/L p-amidinophenyl-methylsulfonyl fluoride (Calbiochem-Behring, La Jolla, Calif). To obtain plasmic digests of crosslinked fibrin in the presence of EGTA, crosslinked fibrin was washed with the buffer with 20 mmol/L EGTA and digested with plasmin solution with 20 mmol/L EGTA.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed[21] using 5% or 7.5% separation gels with 5% stacking gels.[22] Gels were stained for proteins with Coomassie Brilliant Blue R-250 and for carbohydrate by the method of Eckhardt et al.[23]

Case report. The propositus (YN) is a 45-year-old male with mild hypertension. He experienced a brief episode of dizziness and difficulty in walking at the age of 18, sometimes suffered from numbness of extremities since the age of 32, and had angina pectoris since the age of 40.

His paternal grandmother and a paternal aunt died suddenly in their forties without any sign of disease. His father died of cerebral thrombosis and ruptured common iliac artery at the age of 63. Another paternal aunt had hypermenorrhea for a long time and died of acute pneumonia in her forties. The propositus' youngest brother died of acute pneumonia with nasal bleeding and purpura around the mouth at the age of 8 months. The propositus was advised by his home doctor to undergo a blood examination at a local hospital, and was found to have hypofibrinogenemia (71 mg/dL by thrombin time method) with normal PT, PTT, and platelet count. He was then referred to us. His two daughters were also found to have hypofibrinogenemia, but had no history of thrombosis or hemorrhage.

RESULTS

Coagulation studies, including one-stage prothrombin time, activated partial thromboplastin time, factor XIII, antithrombin III, plasminogen, α_2-plasmin inhibitor, and platelet aggregation induced by ADP or collagen, were all within the normal range. As shown in Table 1, thrombin time and reptilase time of the propositus' plasma were markedly prolonged and the plasma fibrinogen concentration evaluated by the thrombin time method was about half the normal value, although the turbidimetric method and immunologic method showed normal levels of plasma fibrinogen. Purified fibrinogen also showed a prolonged thrombin time. The plasma of the two daughters also gave prolonged thrombin and reptilase times and a discrepancy between the plasma fibrinogen concentration assayed by the thrombin time method and that by the turbidimetric or immunologic method (Fig 1).

Release of fibrinopeptide A with thrombin as examined by radioimmunoassay was normal, both with respect to initial rate and total amount. Maximum fibrinopeptide A cleavage from the propositus and normal fibrinogen was 2.09 and 2.03 mol/mol fibrinogen, respectively. Release of fibrinopeptide B as well as of fibrinopeptide A as examined by high-performance liquid chromatography at time intervals after the addition of thrombin also showed no substantial differences between the propositus' fibrinogen and normal fibrinogen. This was also verified by the conversion velocity of Bβ chain to β chain on SDS-PAGE (pictures not shown). A fibrin monomer polymerization curve of the propositus' fibrinogen showed a long lag time (seven minutes *v* two minutes normal) and a decreased slope (ΔOD 350/min = 0.04 *v* 0.138 normal), but the final amplitude was similar to normal (not shown).

Purified fibrinogen was analyzed on SDS-PAGE. Under the reduced condition, the propositus' fibrinogen had an extra protein band with an apparent molecular weight of about 48,000 in addition to the normal γ chain with a molecular weight of 50,000 (γ_{50})[18] (Fig 2, lane 2). This abnormal protein band positively stained for carbohydrate to the same extent as the normal γ chain (Fig 2, lane 4). Densitometric scan of the gel electrophoretogram of the propositus' fibrinogen was performed, and the molar ratio of the Aα chain, Bβ chain, γ chain, and abnormal protein band was calculated as 2 : 1.84 : 1.02 : 1.14 (about 2 : 2 : 1 : 1). No difference in mobility between the propositus' fibrinogen and normal fibrinogen could be detected under the nonreduced condition. These results suggested that this abnormal protein band is a lower molecular weight γ-chain variant. Fibrinogens of two daughters also showed the presence of a lower molecular weight γ-chain variant to the same extent as propositus' fibrinogen on SDS-PAGE (not shown).

To determine whether this abnormal protein band cross-links with activated factor XIII, fibrinogen was treated with

Table 1. Studies for Fibrinogen Function

Sample	Study	Propositus	Normal or Control
Plasma	Thrombin time (sec)		
	without calcium ions	47.6	16.4 to 18.0
	with calcium ions	11.4	6.8 to 8.6
	Reptilase time (sec)		
	without calcium ions	>200	13.3 to 18.4
	with calcium ions	24.7	8.4 to 11.7
	Fibrinogen (mg/dL)		
	Thrombin time method (Clauss)	77	150 to 350
	Turbidimetric method	150	150 to 350
	Immunologic method	210	150 to 350
	Factor XIII (%)	100	80 to 130
Purified Fibrinogen	Thrombin time (sec)		
	without calcium ions	179.0	17.3 to 18.3
	with calcium ions	13.3	6.8 to 7.0
	Release of fibrinopeptides A and B	normal	normal
	Fibrin monomer polymerization	abnormal	normal

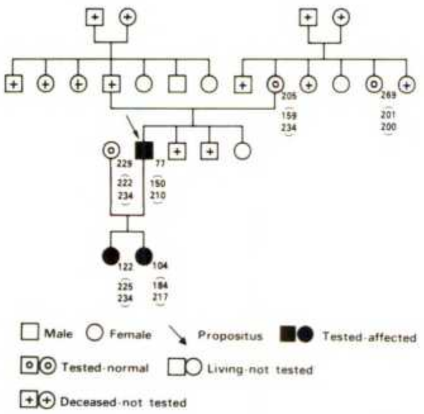

Fig 1. **Pedigree of fibrinogen Kyoto. Figures represent plasma fibrinogen concentrations (mg/dL) measured by the thrombin time method and in parentheses, those determined by turbidimetric (upper) and immunologic methods (lower).**

thrombin and factor XIII in the presence of calcium. The propositus' fibrinogen crosslinked to the same extent as normal; the α chain, γ chain, and the abnormal protein band disappeared, resulting in the appearance of α polymers and g-γ dimers which appeared to exist as three bands but to have smaller molecular weights than γ′-γ′ dimer (Fig 2, lane 6). To make it clear, fibrinogen was purified on DEAE-Sephacel chromatography. A chromatographic profile for propositus' fibrinogen was the same as normal with the appearance of a single type of major peak (peak A,[18] peak 1[24]), and the ascending limb, peak, and descending limb of a major peak showed the presence of low molecular weight γ-chain variant (γ_{Kyoto}) to the same extent on SDS-PAGE (not shown). Application of limited amounts on DEAE-Sephacel made it difficult to obtain enough minor peak (peak C[18], peak 2[24]) for further analysis.

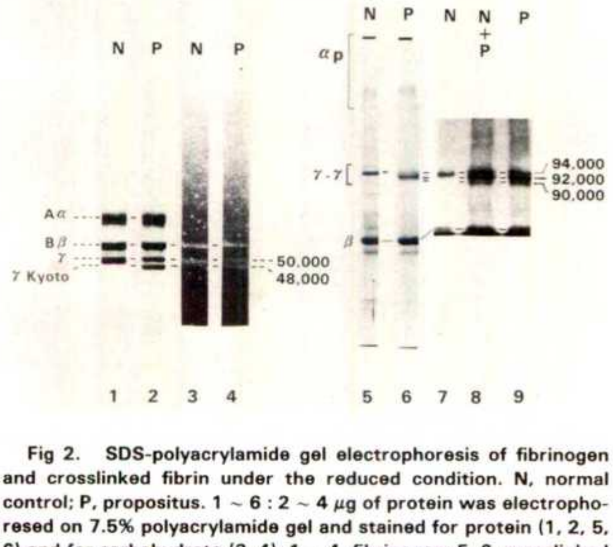

Fig 2. **SDS-polyacrylamide gel electrophoresis of fibrinogen and crosslinked fibrin under the reduced condition. N, normal control; P, propositus. 1 ~ 6 : 2 ~ 4 μg of protein was electrophoresed on 7.5% polyacrylamide gel and stained for protein (1, 2, 5, 6) and for carbohydrate (3, 4). 1 ~ 4, fibrinogen; 5, 6, crosslinked fibrin; 7 ~ 9, crosslinked fibrin prepared from a major peak fibrinogen was electrophoresed on 5% polyacrylamide gel and stained for protein. Only segments of the gel in intensified form are shown for the better understanding of the relation between normal γ-γ dimer and propositus' γ-γ dimers. 7, 2 μg; 9, 8 μg; 8, mixture of 7 and 9. The locations and apparent molecular weights of the chain and dimers are indicated.**

Crosslinked fibrin was prepared from a major peak and electrophoresed on 5% polyacrylamide gel (Fig 2, lanes 7–9). Normal crosslinked fibrin contained only one γ-γ dimer (Fig 2, lane 7) as shown by Francis et al.[18] Crosslinked fibrin of propositus' fibrinogen (Fig 2, lane 9) clearly showed the presence of three γ-γ dimers which had apparent molecular weights of 94,000 (the same as major γ-γ dimer of normal fibrin, Fig 2, lane 8), 92,000, and 90,000, and their molar ratio appeared to be about 1 : 2 : 1, although the three bands which migrated closely did not allow us to measure accurately the intensity of each band densitometrically. This suggested that the three γ-γ dimers are composed of $\gamma_{50} \cdot \gamma_{50}$, $\gamma_{50} \cdot \gamma_{\text{Kyoto}}$, and $\gamma_{\text{Kyoto}} \cdot \gamma_{\text{Kyoto}}$. All three bands were positively stained for carbohydrate (data not shown).

The fragment D dimer obtained from plasmic digestion of crosslinked fibrin in the presence of calcium migrated as a single but somewhat broader band than normal under the nonreduced condition (Fig 3, lane 2), but retained the three γ-γ dimer remnants with apparent molecular weights of 80,000, 77,000, and 74,000 under the reduced condition (Fig 3, lane 4). In contrast, plasmic digestion of crosslinked fibrin in the presence of EGTA resulted in the formation of an identical γ remnant with apparent molecular weight of 25,000 as normal (not shown).

In the following analysis the propositus' fibrinogen could not be separated into two or three populations under the present experimental conditions: The propositus' fibrinogen showed a single precipitation arc with the same migration as normal fibrinogen on immunoelectrophoresis. Fibrinogen bound to fibrinmonomer-Sepharose and was eluted in a single symmetrical peak with the elution buffer. There was no thrombin- or reptilase-nonclottable fibrinogen.

The γ_{Kyoto} cannot be derived from the purification step of

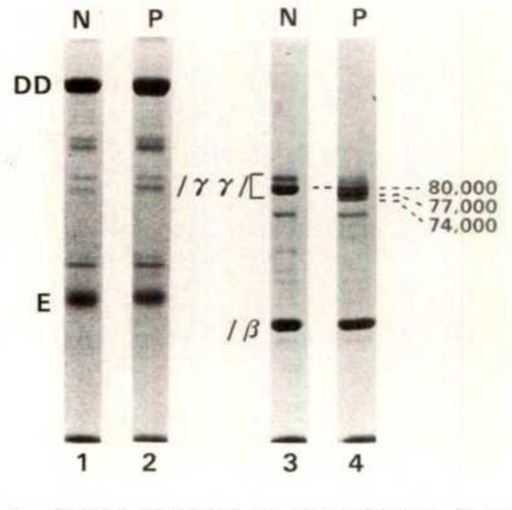

Fig 3. **SDS-polyacrylamide gel electrophoresis of plasmic digests of crosslinked fibrin in the presence of calcium. N, normal control; P, propositus. Eight μg of protein was electrophoresed on 7.5% polyacrylamide gel under nonreduced (1, 2) and reduced (3, 4) conditions. The location of the fragments and apparent molecular weight of γ-γ dimer remnants (/γ-γ/) are indicated. /β, β-chain remnant.**

fibrinogen; the plasma of the propositus and his two daughters was treated with antifibrinogen IgG, or incubated for five minutes at 56 °C. All three immunoprecipitates and heat-precipitates proved to contain γ_{Kyoto} as noted on SDS-PAGE (not shown).

DISCUSSION

The hereditary congenital dysfibrinogenemia described here, designated as fibrinogen Kyoto, is characterized by normal release of fibrinopeptides A and B, defective polymerization of the fibrin monomer, the presence of a γ-chain variant with a lower molecular weight than normal (γ_{Kyoto}), and normal crosslinking ability of γ chains.

A fibrin γ-chain polymerization site has recently been shown to reside in segment Thr-374 to Glu-396,[3] and abnormal fibrinogens with an abnormal γ chain have been shown to have defective fibrin polymerization.[4-8] Fibrinogens Nagoya,[5] Bern I,[6] and Haifa,[7] however, have a normal molecular weight of the γ chain on SDS-PAGE, and fibrinogens Paris I[4] and Grenoble[8] have a larger molecular weight γ chain than normal. Although defective polymerization of fibrin monomer in these abnormal fibrinogens could arise from the abnormalities at or near the γ-chain polymerization site itself, no conclusive arrangement has ever been pinpointed in the γ chain of these abnormal molecules in relation to the impaired function. Only one exception is a recently reported abnormal fibrinogen, fibrinogen Milano I, in which γ-330 Asp has been shown to be substituted by Val.[25] The abnormal protein band found in fibrinogen Kyoto was considered to represent a lower molecular weight γ-chain variant because of (1) its molar ratio of Aα chain: Bβ chain : γ chain : abnormal protein, (2) positive staining for carbohydrate to the same extent as γ chain, and (3) the crosslinking with each other or with the γ chain resulting in the formation of dimers (Fig 2). Crosslinked fibrin contained three types of γ-γ dimers, which shows that crosslinking sites of γ_{Kyoto}, Gln-398 and Lys-406 in normal γ chain, are intact and γ_{Kyoto} has the same crosslinking ability as the normal γ chain. The appearance of three γ-γ dimer remnants in the fragment D dimer of plasmic digests of crosslinked fibrin in the presence of calcium (Fig 3, lane 4) and the disappearance of γ remnants with different molecular weights in plasmic digests of crosslinked fibrin in the presence of EGTA suggest that the COOH-terminal portion of γ_{Kyoto} has shorter peptide chains than normal. If NH_2-terminal portion which is cleaved by plasmin had shorter peptide chains, smaller molecular weight γ-γ dimer remnants could not be retained. These data suggest that a lower molecular weight γ chain, γ_{Kyoto}, comes from a deletion of a certain short segment in γ303-411 of the normal γ chain which has been shown to be cleaved off by plasmin in the presence of EGTA.[26,27] Thus, there is a possibility, though not confirmed, that a certain short segment which is critical for or may affect polymerization is missing in γ_{Kyoto}. This deletion will result in a lower molecular weight γ-chain variant with abnormal fibrin monomer polymerization. A good candidate for the missing segment may be the γ-chain polymerization site, Thr-374 to Glu-396. The possible deletion may correspond to the molecular weight difference of approximately 2,000 between normal γ chain and γ_{Kyoto}. Another candidate for it may be a recently characterized calcium binding site, γ311-336,[28] which is suggested to affect fibrin-monomer polymerization.

Three types of fibrinogen molecules, first discussed for fibrinogen Louisville,[29] are possible in fibrinogen Kyoto as suggested in the case of fibrinogen New York I[30] with a deletion of Bβ (9-72): one with two normal γ chains, one with two γ_{Kyoto}, and one with a normal γ chain and a γ_{Kyoto} chain. However, DEAE-Sephacel chromatography, fibrin monomer-Sepharose chromatography, immunoelectrophoresis, or fibrin clot formation failed to separate the propositus' fibrinogen into two or three main populations. These results leave open the possibility that fibrinogen Kyoto may contain hybrid fibrinogen molecule.

Henschen and Edman[31] identified two minor γ-chain populations in fibrinogen obtained from the pooled plasma of many donors, and showed that the variant isolated in a larger amount is smaller than the main γ chain on gel-filtration chromatography. Although we do not know whether this variant is identical with γ_{Kyoto}, we could not find a γ-chain population with a lower molecular weight in normal fibrinogen obtained from the pooled plasma of at least 20 donors. The cause of sudden death in two family members is not known and three members with γ_{Kyoto} are now free of symptoms. There is a possibility, however, that abnormal fibrinogen Kyoto affects normal hemostasis and that family members who died suddenly without any sign of disease had fibrinogen Kyoto. The propositus' mother and a maternal aunt have normal fibrinogen, and no maternal family members have had a sudden death or hemorrhagic tendency. In summary, a new congenital abnormal fibrinogen Kyoto with impaired fibrin monomer polymerization had a γ-chain variant, γ_{Kyoto}, with a lower molecular weight than normal. Investigation of the precise structural abnormality of γ_{Kyoto} will provide us with additional information on the role of γ chain in fibrin polymerization.

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Granulocyte-Macrophage Colony-Stimulating Factor Enhances Phagocytosis of Bacteria by Human Neutrophils

By Jacob Fleischmann, David W. Golde, Richard H. Weisbart, and Judith C. Gasson

In order to determine whether human granulocyte-macrophage colony-stimulating factor (GM-CSF) can enhance phagocytosis, neutrophils were combined with *Staphylococcus aureus* (*S aureus*), and both the number of bacteria per neutrophil and the percent of neutrophils phagocytizing were assessed in the absence and presence of GM-CSF. Exposure to GM-CSF did not enable neutrophils to ingest unopsonized bacteria. When bacteria were opsonized with serum, both the number of bacteria per neutrophil and the percent of cells phagocytizing were increased by treatment with GM-CSF. Digestion of extracellular organisms by lysostaphin was used to substantiate phagocytosis. These results indicate that another effect of GM-CSF on the mature neutrophil is the enhancement of phagocytosis.
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THE neutrophil or polymorphonuclear leukocyte (PMN) is the major phagocytic cell involved in the first-line defense against invading microorganisms. The neutrophil is also the primary effector cell in acute inflammatory reactions, and participates importantly in autoimmune processes. Recently, human granulocyte-macrophage colony-stimulating factor (GM-CSF), a 22 kd glycoprotein hormone produced by activated T lymphocytes, was shown to have direct effects on the mature PMN. GM-CSF causes neutrophil migration inhibition[1] and primes the PMN to increase superoxide anion generation in response to the bacterial chemoattractant, N-formyl-methionyl-leucyl-phenylalanine (f-MLP)[2] and complement derivatives.[3] We have tested the effect of GM-CSF on PMN phagocytosis, a key function of this cell in host defense.

MATERIALS AND METHODS

Granulocyte-macrophage colony-stimulating factor (GM-CSF). Biosynthetic GM-CSF was purified from medium conditioned by COS monkey cells transfected with the GM-CSF cDNA clone in the p91023(B) expression vector.[4] Purification was performed as described previously[1,5] employing sequential concentration, lentil lectin affinity chromatography, gel filtration, and reverse-phase high-performance liquid chromatography. The concentration of the homogeneous protein was estimated by amino acid analysis, and the purified protein was shown to be free of endotoxin (<0.1 ng/mL) by the limulus amebocyte assay.

Neutrophils (PMN). Purified PMN were obtained from heparinized blood of healthy donors by dextran sedimentation and Hypaque-Ficoll density gradient centrifugation.[6] Purity of the PMN was ≥95% by Giemsa stain and viability of neutrophils at each time point, assessed by trypan blue exclusion, was ≥97%.

Organisms. *Staphylococcus aureus* ATCC 29213 (SA) was grown on trypticase soy agar (TSA) slants and kept at 4 °C. For individual experiments, the organism was grown overnight in trypticase soy broth (TSB) at 37 °C, washed twice by centrifugation, and suspended in Hank's balanced salt solution with 5 mmol/L Hepes, at pH 7.4 (HBSS). The desired concentration was attained by monitoring spectrophotometric absorbance at 600 nm, using an appropriate optical density/colony-forming units (CFU) curve.

Serum. Serum was obtained from an AB donor and kept at −70 °C until used. For opsonization, either freshly thawed (NS) or heat-inactivated serum (HIS) (56 °C for 30 minutes) was used. Bacteria (1×10^9) were opsonized at 37 °C for 30 minutes in one mL of serum, washed twice, and resuspended in HBSS.

Lymphokine stimulation of neutrophils. PMN (2×10^6/mL) were incubated in HBSS with 0.01% bovine-serum albumin (BSA) at 37 °C in the presence of either 100 pmol/L or 1.0 nmol/L GM-CSF. For sham stimulation, cells were incubated in the same buffer in the presence of the protein diluent (PBS with 0.01% BSA) only. After either 15 minutes or 120 minutes, the cells were washed with centrifugation and resuspended in HBSS.

Phagocytosis assay. PMN (1×10^6/mL) and SA (1×10^8/mL) were combined in a final volume of one mL in 12 mm × 75 mm sterile, capped plastic tubes and rotated end over end at 37 °C. At appropriate times, 0.2 mL of assay solution was removed and combined with 0.2 mL of HBSS with 0.2 mmol/L N-ethyl maleimide (NEM) to inhibit additional phagocytosis.[7] Cytocentrifuge slides were prepared, fixed with methanol, stained with Giemsa, and examined by light microscopy. The slides were evaluated blind by masking the identifying information. Individual PMN were evaluated as having either 0, 1 to 10, 11 to 20, 21 to 30, or >30 bacteria associated with them. A weighted phagocytic index (WPI) was calculated by multiplying the number of PMN in each category by 0, 1, 2, 3, 4, respectively, and dividing the total score by the number of neutrophils examined (usually 100). The percentage of PMN phagocytizing (having one or more bacteria) was also assessed.

Lysostaphin modification. As light microscopy does not differentiate between surface adherence and phagocytosis, lysostaphin was used in one assay to digest away extracellular bacteria.[8] Cells and bacteria were combined and rotated as above. At appropriate times, 0.2 mL of the mixture were removed, combined with 0.2 mL of HBSS containing 0.2 mmol/L NEM and 40 U/mL of lysostaphin and incubated at 37 °C for 30 minutes. The mixture was then chilled in an ice water bath and cytocentrifuge slides prepared, fixed, stained, and evaluated as above. Prior to assay, 20 U/mL lysosta-

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phin reduced colony counts of SA from 1 × 10^8 to 0 in 30 minutes, even in the presence of 0.1 mmol/L NEM. Furthermore, the regular stained slides showed many free bacteria; cultures with lysostaphin showed only rare extracellular bacteria.

Intracellular killing assay. A modification of an assay described by Quie was used.[9] Briefly, PMN stimulation by GM-CSF was performed as above. Unopsonized SA and PMN were combined, both at 2 × 10^6/mL in preheated (37 °C) HBSS containing 10% NS, and rotated end over end at 37 °C. At appropriate times, 0.1 mL of mixture was removed, serially diluted in HBSS, and plated in duplicates on TSA. Plates were incubated at 37 °C and counted.

RESULTS

Neutrophils combined with untreated SA were inefficient in phagocytizing bacteria, and stimulation with GM-CSF showed no effect (Table 1). Figure 1 shows the combined, normalized data regarding phagocytosis of opsonized SA. Enhancement of phagocytosis was observed after 15 minutes of exposure of PMN to 100 pM GM-CSF (Fig 1A). An even greater increase over control was observed after 120 minutes of preincubation ($P < .05$; Fig 1B), a concentration and time period observed to enhance other PMN functions.[2,3] Raw data from one experiment are shown for comparison (Table 1). A tenfold increase in GM-CSF concentration to 1 nmol/L did not further increase this priming effect (Fig 1C).

In order to distinguish between phagocytosis of the bacteria and adherence to the outer surface of the neutrophil membrane, lysostaphin treatment was performed. The organisms were resistant to digestion with lysostaphin, demonstrating the apparently phagocytosed bacteria were in fact intracellular (Fig 1D). Another measurement of phagocytosis is the percent of PMN-ingesting organisms. As shown in Fig 2, after exposure to 100 pM GM-CSF for two hours, a higher percentage of PMN was phagocytic, as compared to control.

Opsonization with normal serum and heat-inactivated serum was compared to determine whether heat-labile complement components were required. Figure 3 shows that priming by GM-CSF enhanced phagocytosis of organisms opsonized with heat-inactivated serum (part B), but was more efficient when both heat-labile and heat-stable components were available (Part A).

Table 1. Enhancement of Phagocytosis by GM-CSF

Time*	Neutrophil† GM-CSF Stimulation	Bacteria‡ Opsonization	Bacteria per Neutrophil 0	1-10	11-20	21-30	>30
4 min	–	–	97	3	—	—	—
16 min	–	–	96	4	—	—	—
32 min	–	–	97	3	—	—	—
4 min	+	–	96	4	—	—	—
16 min	+	–	99	1	—	—	—
32 min	+	–	98	2	—	—	—
4 min	–	+	60	39	1	—	—
16 min	–	+	40	46	11	3	—
32 min	–	+	44	25	23	5	3
4 min	+	+	37	60	3	—	—
16 min	+	+	12	46	26	10	6
32 min	+	+	13	19	38	19	11

*Time before phagocytosis was inhibited.

†Exposure was to 0.1 nmol/L GM-CSF (+) or diluent (–) for two hours.

‡Opsonized with normal AB serum for 30 minutes.

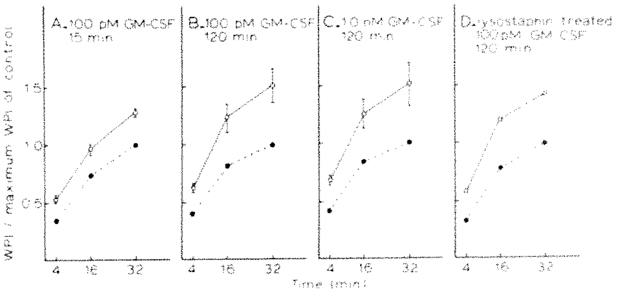

Fig 1. Phagocytosis of *S. aureus* by PMN exposed to diluent only (control) (●) or exposed to GM-CSF (○) prior to combination with bacteria. WPI (weighted phagocytic index; see Methods) were calculated for each time period (see text) and expressed as a ratio of maximum WPI of control (WPI at 32 minutes). Error bars represent SEM. At 100 pmol/L GM-CSF for 15 minutes (A, n = 2), the increases are not yet significant, but after 120 minutes (B, n = 4), all time points are significant at $P < .05$ (as calculated by *t* test for comparison of paired data). Tenfold increase of GM-CSM (C, n = 3) did not further enhance phagocytosis. In one experiment with lysostaphin digestion (D) to prove ingestion (see text), increases paralleled those of B.

Figure 4 shows the data regarding intracellular killing by neutrophils. In this assay, neutrophils are combined with bacteria in a ratio of 1:1, thus obviating any effects of GM-CSF on phagocytosis. There is no difference in killing between GM-CSF–stimulated and –unstimulated cells.

DISCUSSION

In addition to stimulating hematopoietic cell proliferation, colony-stimulating factors affect the functional activity of mature effector-cells.[10-13] When murine peritoneal macrophages are exposed to GM-CSF, they increase their ability to phagocitize and kill Leishmania tropica.[14] Conditioned media with colony-stimulating activity from several cell types have also been shown to increase tumoricidal capacity[15]

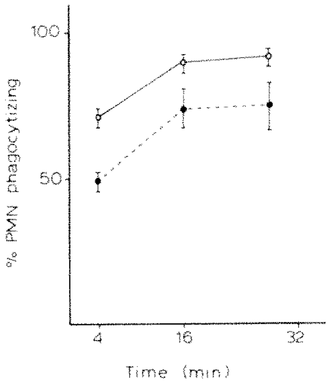

Fig 2. Percent of PMN having ingested one or more organisms. PMN were exposed to diluent only (control) (●) or 100 pmol GM-CSF (○) for 120 minutes (n = 4). Error bars represent SEM. At all time points, more GM-CSF-treated PMN contained bacteria; the values at the earliest times tested were significantly different (at 4 min, $P < .05$, as calculated by student *t* test).

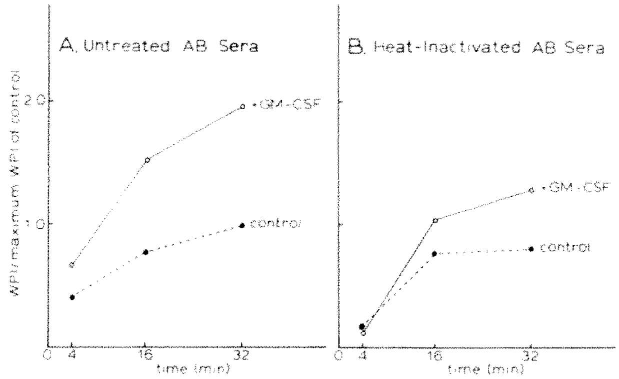

Fig 3. **Comparison of (A) untreated and (B) heat-inactivated (56 °C for 30 minutes) sera as sources of opsonin. PMN were exposed to 1 nmol/L GM-CSF or diluent (control) for 120 minutes prior to combination with bacteria.**

and intracellular killing of Candida parapsilosis[16] by mononuclear phagocytes. Similar end-cell regulatory activity by GM-CSF has been demonstrated for murine and human PMN. GM-CSF increases antibody-dependent cytotoxicity,[17] inhibits PMN migration,[1] and enhances superoxide anion generation in response to f-MLP[2] and C5a.[3] The present results extend these observations to include PMN phagocytic capacity.

GM-CSF did not induce phagocytosis of unopsonized bacteria, but primed PMN to be more efficient when presented with bacteria already opsonized by serum. These findings are very similar to those of PMN and f-MLP interaction. GM-CSF by itself did not stimulate superoxide anion generation, but primed the cell to increase its response to f-MLP by fourfold.[2] The concentration of GM-CSF (100 pmol/L) and time of exposure (two hours) at which maximal effects were generated were similar in both cases.

Opsonins in serum needed for *S aureus* ingestion include antibody and complement components.[18] It appears that GM-CSF is able to enhance phagocytosis mediated by both opsonins. When heat-inactivated serum was used (Fig 3B), GM-CSF enhanced uptake of bacteria, but when both antibody and complement were present (Fig 3A), this enhancement was more marked. The mechanism by which GM-CSF enhances PMN phagocytosis is not known. One possible explanation is modulation of receptors on the cell for the known opsonins, IgG and C3. Indeed, a lymphokine produced by the Mo cell line (the original source of this GM-CSF) has been shown to activate C3 receptors on murine peritoneal macrophages.[19] Intracellular killing of *S aureus* was not increased by GM-CSF stimulation (Fig 4), but this organism is highly susceptible to the bacteriocidal activities of PMN. Augmented microbiocidal activity may be demonstrable with other organisms.

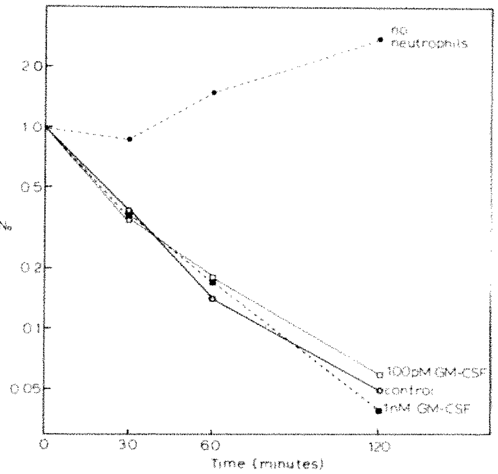

Fig 4. **Intracellular killing of *S. aureus* by PMN. N, number of colonies at each time interval; No, number of colonies at time of initial combination of cells and bacteria.**

GM-CSF has been shown to be produced by activated T lymphocytes[4] and present in areas of inflammation.[13] The biological activities of GM-CSF include inhibition of neutrophil migration, enhanced phagocytosis, and increased production of bacteriocidal metabolites, suggesting an important role for this mediator in host defense.

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Biological and Immunological Characterization of ATG and ALG

By Eric L. Raefsky, Pedro Gascon, Alois Gratwohl, Bruno Speck, and Neal S. Young

Antithymocyte globulin (ATG) and antilymphocyte globulin (ALG) are effective therapies in aplastic anemia; their mechanism of action is undefined. We assayed multiple properties of ATG and ALG to address the biological and immunological bases for differences between ATG and ALG and lot variation. In addition, we studied a lot reported to be inactive in an American clinical trial; however in retrospect, this lot appeared to be active in patients treated in Europe. Immunoprecipitation of thymocyte and lymphocyte membrane proteins with ATG and ALG showed between 14 and 18 major bands on SDS-PAGE, but the patterns for ATG and ALG were not identical. The ability of ATG and ALG to block binding of labeled monoclonal antibodies was assessed using flow cytometry and a radioimmunoassay. In general, there was more lot variation among ALGs than ATGs; however, all ALG lots were more potent blockers of binding of anti-HLA-DR and anti-Leu 1 antibodies than was ATG. Both ALG and ATG effectively blocked binding of anti-Leu 2a, anti-Leu 3a, anti-Leu 4, anti-Leu 5b, and anti-IL 2 receptor abs; neither blocked binding of anti-Leu 7. All preparations were capable of inducing T-cell blastogenesis, although there was considerable lot variation. All lots lysed 60% to 75% T cells in a rabbit complement-mediated cytotoxicity assay, with most having a plateau of activity at 5 to 10 ug/mL. Two lots of ALG, including the lot reported to be clinically inactive, showed less toxicity at suboptimal concentrations and did not plateau even at 80 ug/mL. In total, these results indicate important differences between ATG and ALG in general, more lot variation among ALGs than ATGs and only differences in cytotoxicity between an "inactive" lot of ALG and most, but not all, other active ATG and ALG preparations.

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IMMUNE horse sera prepared against human lymphocytes (ALG) and thymocytes (ATG) have—surprisingly—been shown to be effective therapy for aplastic anemia, resulting in transfusion independence in 40% to 80% of patients.[1-6] The discovery of the usefulness of ALG was based empirically on clinical studies and not dependent on knowledge of the precise etiology of bone marrow failure.[7] A likely mechanism of action of ATG and ALG is immunosuppression, as some proportion of cases of aplastic anemia appear to be immune mediated[8-10]; however, ATG also has been shown to stimulate T-cell proliferation and lymphokine production,[11] and a direct effect on hematopoietic progenitors has been inferred by some investigators.[12-14] Without an established mechanism of action for ATG and ALG, prospective testing of horse sera for clinical activity in aplastic anemia has not had a firm rational basis.

Despite their effectiveness, there are intrinsic difficulties in using animal sera as treatment, including the broad specificity of these antisera for human cells,[15] allergic reactions like serum sickness,[16] important differences in their methods of production, uncertain formulations, and potential lot variation. In fact, one batch of ALG (lot 97412) has been reported to be clinically ineffective,[17] raising the issue of significant lot variation. Because of the practical and theoretical uncertainties concerning the constitution of ALG and ATG, we undertook to characterize in detail this lot and other preparations using a variety of biological and immunological assays. Special emphasis was placed on differences between ATG and ALG in general, lot variation within each type of horse serum preparation, and laboratory correlates of clinical acvtivity.

MATERIALS AND METHODS

ATG and ALG preparations. ATG lots 17924, 844, and 731 were provided by the Upjohn Company (Kalamazoo, Mich) and ALG lots 160, 162, 15343, 181 S, and 97412 by the Swiss Serum and Vaccine Institute (Berne, Switzerland) as sterile solutions at 50 mg/mL in saline. The protein concentration was confirmed spectrophotometrically and the preparations determined to be greater than 95% IgG by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomasie blue staining.

ATG was prepared by injecting horses with homogenized emulsions of normal thymocytes removed from children undergoing cardiac surgery.[18] Antibodies to RBC stroma and human plasma proteins were removed by absorption before the gamma globulin was purified. All ATG lots represent pooled sera from four inoculated animals, obtained and processed at different times. ALG lot 17924 has been successfully employed in clinical trials with response rates to transfusion independence in severe disease of about 40%[19] (and the NHLBI multicenter trial, Young N, unpublished data). ALG EC162 and 15343 were prepared using fresh whole lymph from an adult patient undergoing therapeutic thoracic duct drainage; plasma from 2 horses were pooled before purification. The preparation of one ALG lot differed in that thoracic duct mononuclear cells which had been cryopreserved for 3 years were purified by Ficoll-Hypaque sedimentation before being used as the immunogen; the same factory lot (170S) was split to provide ALG for European use (ALG 92125) and American studies (ALG 97412) (N. Chariatte, Swiss Serum Institute, personal communication). ALG 181S resulted from the immunization of new horses with fresh lymph from a new source. The production and clinical utility of these ALG preparations are summarized in Table 1; details of the clinical trials from Basel have been published elsewhere.[5]

Preparation of peripheral blood cells. Peripheral blood was drawn into syringes containing 50 u/mL preservative-free heparin (Jones, McNeal, and Feldman, St. Louis, Mo). Peripheral blood mononuclear cells (PBMNC) were obtained by Ficoll-Hypaque

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Table 1. Preparation and Clinical Results of ALG Lots

Lot	EC162	15343	92125 (97412)	181S
Production date	5/78	2/80	8/82	1/84
Horses immunized	Vulcan III	Vulcan III Peter I	Vulcan III Peter I	Vulcan IV Peter II
Antigen	donor: VB concentrated lymph	donor: VB concentrated lymph	donor: VB Ficoll-separated lymphocytes cryopreserved 3 yr	donor: WG concentrated lymph
No. Patients treated	29*	28†	12†	17†
Age (median)	16	14	23	31
No. Severe disease‡	29	20	6	11
No. Acute disease	29‡	15§	8§	11§
No. Alive and transfusion independent (%)‖	20 (69%)	23 (82%)	9 (75%)	23 (71%)

*Clinical results of lot EC162 from reference 4.

†Clinical results of patients treated in Basel.

‡As defined in reference 4. Diagnosis less than 1 month prior to referral.

§Treatment within 2 months of diagnosis.

‖Improvement in blood counts so that patient's hematologic status no longer qualified as severe and blood product transfusion was not needed by 4 months after initiation of ALG therapy.

density sedimentation, washed three times, and resuspended in appropriate media. T cells were fractionated by rosetting with neuraminidase treated sheep red blood cells overnight at 4 °C. Phytohemagglutinin (PHA)–stimulated peripheral blood mononuclear cells (PHA-PBMNC) were obtained by incubating PBMNC at an initial cell concentration of 1.5 × 10^6 cells/mL with PHA (GIBCO, Grand Island, NY) at a final concentration of 10 ug/mL in RPMI 1640 supplemented with 2 mmol/L glutamine, 20% heat-inactivated fetal calf serum (Hyclone, Logan, Utah), and antibiotics at 37 °C in a 5% CO_2 humidified chamber. After 48 hours, the cells were washed, resuspended in the same media without PHA but supplemented with 10% partially purified T-cell growth factor (Cellular Products, Portland, ME), and incubated for an additional 48 to 72 hours before harvesting. Thymocytes were obtained from a child undergoing cardiac surgery, frozen in media containing 10% dimethylsulfoxide, and stored in a liquid nitrogen freezer until used.

Cytotoxicity assays. T cells were labeled with 200 μL of ^{51}Cr (1 mCi/mL solution, Amersham, Arlington Heights, Ill) per 1 × 10^7 cells for one hour at 37 °C, washed, and resuspended in RPMI plus 0.1% BSA at a concentration of 20,000 cpm/75 uL. Seventy-five uL of freshly prepared dilutions of heat inactivated ATG, ALG, or nonimmune horse IgG (HIgG, United States Biochemical Corp, Cleveland, OH) was added to 75 uL of labeled cells in triplicate and the cells were incubated for 30 minutes at room temperature. Rabbit complement (Cedarlane, Hornsby, Ontario) at an optimal final dilution of 1:6 or human anti-AB antibody negative serum at a final concentration of 1:24 was then added and the incubation was continued for an additional 60 minutes at 37 °C. The cells were then centrifuged and cytotoxicity determined by assaying the supernatant for ^{51}Cr release.

Blastogenesis assay. The ability of ATG and ALG to stimulate blastogenesis of PBMNC was assayed by measuring DNA synthesis by methyl ^{3}H-thymidine incorporation as previously described.[11] The stimulatory index (SI) was computed as the ratio of induced to spontaneous isotope incorporation. In 2 experiments, the proliferating cells were determined by analyzing by flow microfluorometry the proportion of activated T cells (Leu 4^+, IL 2 receptor$^+$) and B cells (Leu 12^+) before and after simulation.

Flow microfluorometry analysis. For blocking experiments, PBMNC or PHA-PBMNC were washed and resuspended in Hanks balanced salt solution (HBSS; M.A. Bioproducts, Walkersville, Md) supplemented with 0.1% bovine serum albumin (BSA) and 0.1% sodium azide (NaN_3). 1 × 10^6 cells were preincubated with 100 μL of HIgG, ATG, ALG, or media in varying concentrations for 30 minutes at 4 °C, washed twice, and then incubated with 5 uL of commercially available monoclonal antibodies or control IgG directly conjugated to fluorescein (FITC-Ab, Becton Dickinson, Sunnyvale, Calif) for an additional 30 minutes at 4 °C in the dark. The cells were washed twice, resuspended in HBSS plus 0.1% BSA and 0.1% NaN_3, and analyzed by flow microflourometry using an Epics V (Coulter, Hialeah, Fla). Flourescence was measured using a logarithmic scale. Using the IMMUNO program of the EASY 88 Computer System (Coulter), negative cells in the control samples (ie, samples preincubated with media alone before labeled with FITC-Ab) were subtracted from the corresponding fluorescent channel from the samples preincubated with ATG or ALG. This program therefore effects a decrease in mean fluorescence proportional to the blocking of the cell surface protein by the preincubation preparation, but does not alter the proportion of cells scored as positive by comparison to control (nonimmune mouse monoclonal antibody) preparations. Extrapolation from these data allowed an estimation of the amount of the preincubation preparation needed for 50% blocking of each FITC-Ab. In most cases, preincubation with the highest concentration of HIgG resulted in less than 5% blocking; in the occassional case in which this nonspecific blocking was greater than 5%, the mean fluorescence of the sample preincubated with HIgG was used as the control.

^{3}H-anti-Tac blocking studies. The ability of ATG, ALG, and HIgG to block a ^{3}H-monoclonal antibody directed against the interleukin-2 (IL 2) receptor (^{3}H-anti-Tac) from binding to HUT 102 cells (which contain approximately 300,000 IL-2 receptors per cell[20]) was determined using a modification of a previously described assay.[21] 10^6 HUT 102 cells were washed in PBS and resuspended in binding media as previously described.[21] Cells were incubated with varying dilutions of unlabeled anti-Tac, HIgG, ATG, or ALG for two hours at room temperature in duplicate. 5.5 ng of ^{3}H-anti-Tac (equivalent to 15,000 cpm, kindly supplied by Dr Thomas Waldmann) was added and the cells incubated for an additional 60 minutes at room temperature. Cell bound radioactivity was determined after the reaction mixture was centrifuged through a 1 mol/L sucrose cushion and the supernatant aspirated. Unbound ^{3}H-anti-Tac was determined by subtracting antibody bound from total antibody added.

Cell surface iodination, immunoprecipitation, and electrophoresis. 10^8 cells were washed and resuspended in Dulbecco's phosphate-buffered saline without calcium or magnesium (DPBS; GIBCO) and the cell surface proteins radiodinated with 2 mCi of ^{125}I-sodium iodide (New England Nuclear, Boston, Mass) via the lactoperoxidase-H_2O_2 method.[22] Cells were washed 3 times in DPBS containing 1 mmol/L phenylmethylsulfonylfluoride (PMSF) and 1 mmol/L diisopropylflourophosphate (DFP, both from Sigma, St. Louis, Mo) and the iodinated proteins solubilized by lysing the cells in 1 mL of DPBS containing 1 mmol/L PMSF, 1 mmol/L DFP, and 0.5% NP-40 for 30 minutes at 4 °C followed by centrifugation to remove cellular debris. To immunoprecipitate cell surface proteins with ATG and ALG, 50 uL of solubilized iodinated lysate from PHA-PBMNC or thymocytes were precleared twice with 300 uL of 10% Staph protein A (Immunoprecipitin, Bethesda Research Labs, Bethesda, Md) and then incubated with 20 uL of antibody lots at a concentration of 50 mg/mL overnight at 4 °C. Bound lysate was immunoprecipitated with Staph protein A, washed 3 times in DPBS containing 0.1% BSA, 0.02% sodium azide, 0.5% NP-40, and 0.01% sodium dodecyl sulfate (SDS, Bio-Rad Laboratories, Richmond, Calif), and resuspended in a reducing sample buffer according to Laemmli.[23] In preliminary experiments, comparable results were obtained using Staph protein A and rabbit antihorse immunoglobulin coated Staph protein A. Final pellets were boiled, Staph protein A removed by centrifugation and the samples subjected to SDS-polyacrylamide gel electrophoresis using 5% to 15% discontinuous linear gradient gels. After electrophoresis, the gels were fixed, and fluorographed for 1 to 10 days at −70 °C using intensifier screens and Kodak X-O-Mat film.

RESULTS

Immunoprecipitation of cell surface membrane proteins. To determine which cell surface proteins were immunoprecipitated by ATG and ALG, cells similar to the immunogens used for the preparation of ATG and ALG were employed. Both ATG and ALG precipitated a discrete number of major protein bands from membranes of both PHA-PBMNC (Fig 1) and thymocytes (data not shown), without apparent major differences in results using the two cell types. Both ATG and ALG preparations consistently precipitated several major protein components, for example, proteins of molecular weight 182,000, 138,000, 112,000, 93,000, 43,000, and 35,000. However, other protein components were better or exclusively precipitated by one or the other preparation: for example, ATG uniquely precipitated bands of 295,000 and 30,000 and ALG a band of 166,000. Two lots of ATG (844 and 17924) had identical patterns of immunoprecipitation of thymocyte and PHA-PBMNC surface proteins. In contrast, there was variability in the number and intensity of proteins immunoprecipitated by different ALG preparations (lots EC162, 15343, and 97412).

Definition of antibody specificities. In order to define specific antibody specificites in horse sera, the binding of fluoresceinated monoclonal antibodies to T-cell antigens was quantitated after preincubation of cells with ATG or ALG. A typical experiment, illustrated in Fig 2a, shows that increasing concentrations of ATG 844 progressively inhibited binding of anti-Leu 4-FITC as demonstrated by a decrease in mean fluorescence. The blocking ability of different lots of ATG and ALG for anti-Leu 4-FITC varied (Fig 2b), presumably reflecting differences in the concentration of this antibody specifity within these preparations.

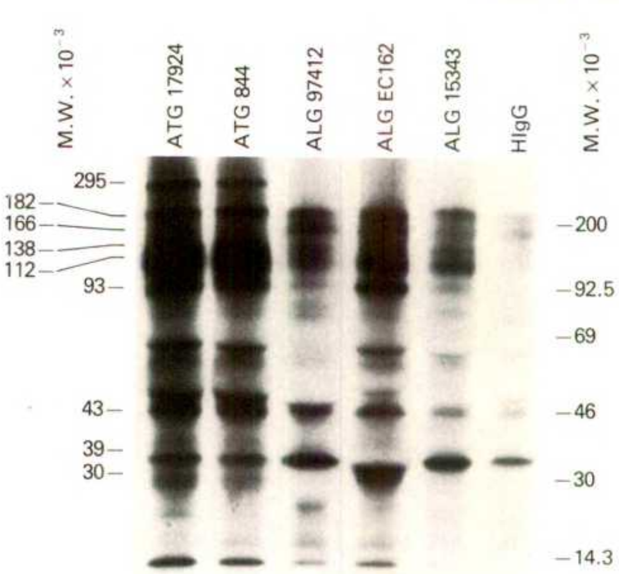

Fig 1. Immunoprecipitation of ^{125}I-labeled PHA-PBMNC membrane lysate by ATG 19724 (lane 1); ATG 844 (lane 2); ALG 97412 (lane 3); ALG EC162 (lane 4); ALG 15343 (lane 5); and HIgG (lane 6). Positions of molecular weight markers are shown on the right and the molecular weights of bands referred to in the text on the left.

Extrapolation from these data allowed an estimation of the concentration of ATG, ALG, or unlabeled monoclonal antibody needed for a 50% decrease in mean fluorescence (Table 2). Comparison of the relative blocking ability for the various antibodies is complicated by differences in antigen quantity on the cell surface, unknown antibody affinities for antigens, and the likelihood of significant interactions among antibodies of differing specificity. However, these results do allow a quantitative comparison of different preparations of ATG and ALG for each monoclonal antibody. Comparison of an ATG lot (844) and an ALG lot (EC162) showed similar amounts of blocking ability for antigens recognized by monoclonal antibodies to Leu 2a, Leu 3a, Leu 4, and Leu 5b. However, ALG EC162 was over 30 times more potent than ATG 844 in blocking anti-Leu 1 (50% inhibition concentration: 5.8 mg/mL *v* 0.16 mg/mL) and more than 20 times more potent in blocking anti-HLA-DR binding (50% inhibition concentration: 104 mg/mL *v* 4.7 mg/mL).

These differences in the blocking ability of ATG and ALG were not due to lot variation. The 50% blocking concentrations for ATG lots 17924 and 844 were similar, with less than twofold variation for all monoclonal antibodies tested (data not shown). Parallel to the immunoprecipitation data, however, the differences among different ALG lots were more pronounced. ALG EC162 was more potent than ALG 97412 in blocking antibody binding to Leu 1 (4 x), Leu 2a (3 x), Leu 4 (4 x), and Leu 5b (3 x); these preparations were similar in their ability to block binding of anti-Leu 3a and anti-HLA-DR. In general, lot 15343 was more similar to 97412 than to EC162. However, even the least potent ALG lot was still 10 times more potent than ATG in preventing the binding of anti-Leu 1 and anti-HLA-DR antibodies.

This analysis was not able to demonstrate antibodies in ATG or ALG able to block the binding of monoclonal

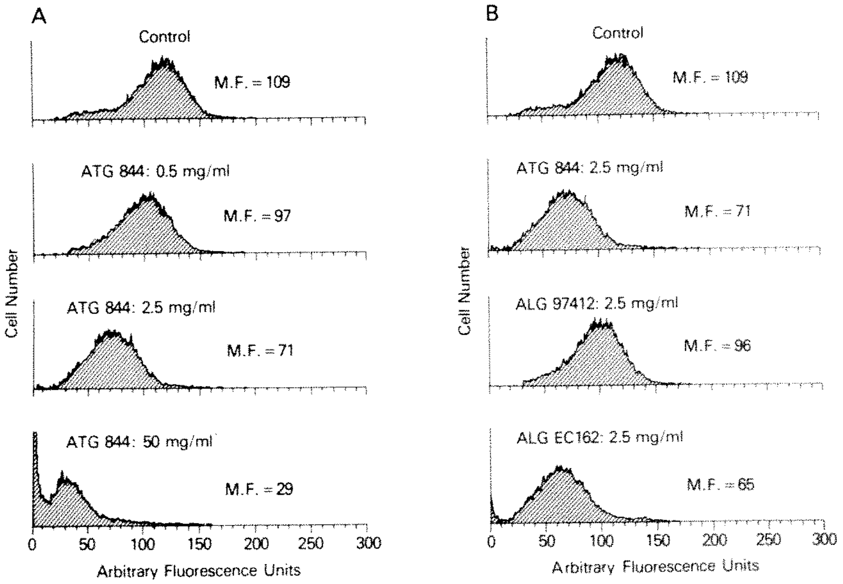

Fig 2. Flow microflourometry blocking study of anti-Leu 4 ab. (A) Binding of anti-Leu 4-FITC to PBMNC after preincubation with media alone (control, upper panel) or increasing concentrations of ATG 844 was analyzed by flow microflourometry in this representative example. Only the cells that were "positive" by the IMMUNO program are shown. (B) Binding of anti-Leu 4-FITC to PBMNC after preincubation with media alone (control, upper panel) or various lots of ATG or ALG at the same concentration. The decrease in mean flourescence (MF) is proportional to the relative blocking ability of the different preparations.

antibodies to Leu 7 (a marker for large granular lymphocytes) or the interleukin 2 receptor (Table 2). However, as both thymocytes and thoracic duct lymphocytes include cells that express the interleukin-2 receptor,[26-29] a more sensitive assay evaluating the ability of these horse immunoglobulin preparations to block the binding of a tritiated monoclonal antibody reactive with the interleukin-2 receptor (called anti-Tac) to HUT 102 cells was performed (Fig 3). Under these conditions, both ATG and ALG were able to significantly block the binding of ^{3}H-anti-Tac with equal potency. The potency of ATGs and ALGs compared to the specific anti-Tac monoclonal antibody (about 1000 x) was similar to the relative potency of the antisera compared to other monoclonal antibodies (Table 2). The explanation for the discrepancy between the behavior of ATGs and ALGs in the radioimmunoassay and the flow cytometry blocking experiments is unclear, but may reflect the greater density of Tac antigen on the surface of the malignant cell line HUT 102 compared to PHA-PBMNC[20] and the use of different monoclonal antibodies directed against the interleukin-2 receptor which were employed in the two assays.

Blastogenesis. The mechanism of action of ATG and ALG in the treatment of aplastic anemia is unknown; investigators have shown both immunostimulatory and cytotoxic properties of these preparations. The ability of several ATG and ALG lots to induce the blastogenesis of PBMNC was measured as a general indicator of T-cell stimulatory potential (Table 3). Although all preparations were able to induce blastogenesis, there was variation among different ATG and ALG lots. Neither ATGs nor ALGs were uniformly superior as a class of sera. There was no correlation between activity in the blastogenesis assay and antibody specificity directed to Leu 4, an antigen required for mitogen activation of lymphocytes. Using nonadherent bone marrow cells as target populations, both ATGs and ALGs were also able to stimulate hematopoietin release from PBMNC to approximately the same degree (data not shown). At higher horse sera concentrations (greater than 10 ug/mL), blasto-

Table 2. Comparison of Antibody Specificities in ATG and ALG

		50% Blocking Concentration (mg/mL)†				
FITC-Ab	WHO "CD" Classification‡	Unlabeled Antibody	ATG 844	ALG EC162	ALG 97412	ALG 15343
Anti-Leu 1 (pan-T cell antigen)	CD5	0.0001	5.8 ± 1.1	0.16 ± 0.06	0.61 ± 0.18	0.71 ± 0.25
Anti-Leu 2a (suppressor/cytotoxic T cells)	CD8	0.0020	0.83 ± 0.34	0.50 ± 0.33	1.5 ± 0.8	0.98 ± 0.40
Anti-Leu 3a (helper T Cells)	CD4	0.0001	1.6 ± 0.7	1.5 ± 0.8	3.9 ± 1.7	4.2 ± 0.5
Anti-Leu 4 (T-cell receptor-associated antigen)	CD3	0.0010	3.8 ± 1.5	2.9 ± 0.9	10.3 ± 3.0	9.7 ± 6.6
Anti-Leu 5b (E-rosette receptor)	CD2	0.0001	0.24 ± 0.06	0.18 ± 0.06	0.49 ± 0.04	0.48 ± 0.02
Anti-leu 7 (LGL, NK cells)	—	0.0400	*	*	*	NT
Anti-HLA-DR (activated T cells)	DR	0.0100	104 ± 77	4.7 ± 2.5	7.8 ± 5.8	10.0 ± 8.6
Anti-IL 2 Receptor	CD25	0.0003	*	*	*	NT

WHO, World Health Organization; LGL, large granular lymphocytes; NK, natural killer; NT, not tested.

*Less than 10% blocking with the highest dilution (50 mg/mL) of ATG/ALG.

†Values represent the mean ± SD of the extrapolated concentration of ATG, ALG, or unlabeled antibody in 2 to 6 determinations that resulted in a 50% decrease in FITC-Ab mean fluorescence.

‡Cluster designation of antigens as defined by the First and Second International Workshop on Human Leucocyte Differentiation Antigens (reference 24 and 25).

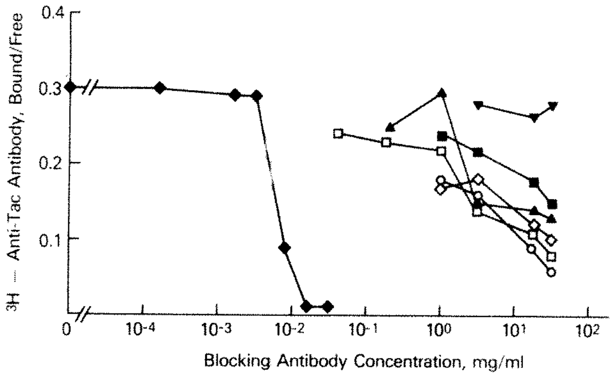

Fig 3. Binding of ^{3}H-anti-Tac to HUT 102 cells after preincubation with unlabeled anti-Tac antibody (◆); HIgG (▼); ATG 844 (■); ATG 17924 (▲); ALG 97412 (○); ALG EC162 (□); and ALG 181S (◇). One of 3 representative experiments is shown.

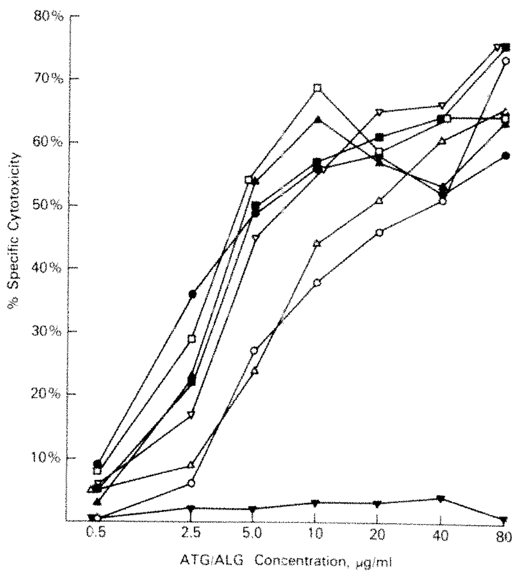

Fig 4. Cytotoxicity of E-rosetted cells (greater than 90% positive T cells by flow microflourometry analysis) with complement and various dilutions of HIgG (▼); ATG 844 (■); ATG 17924 (▲); ATG 731 (●); ALG 97412 (○); ALG EC162 (□); ALG 181S (△); and ALG 15343 (▽). Each point represents the mean of 3 paired experiments. The SEM of each point was less than 10%.

genesis was often inhibited, even though cells were still viable. This effect may have been due to the blocking of the interleukin-2 receptor at these concentrations.

Cytotoxicity. All preparations of ATG and ALG tested were effective in a complement-dependent cytotoxicity assay using T cells as targets and rabbit serum as a source of complement (Fig 4). All lots of ATG and ALG had a peak cytotoxicity of 60% to 75%, with most having a plateau concentration between 5.0 to 10.0 ug/mL; significant lysis was not demonstrable at concentrations below 0.5 ug/mL. Although ALGs 97412 and 181S had peak cytotoxicity values comparable to other lots tested, they differed in that a plateau was never reached over the range of concentrations tested. At suboptimal concentrations (2.5 to 10 ug/mL), these same lots were significantly less cytotoxic than the other ATG and ALG preparations tested. Similar results were obtained using human serum as a source of complement, except 10 to 15 times higher concentrations of horse sera were needed for similar degrees of cytotoxicity (data not shown).

Table 3. Summary of ATG- and ALG-Induced Blastogenesis of Peripheral Blood Mononuclear Cells from Patients with Aplastic Anemia*

	n	Relative Blastogenesis†
ATG Lot		
731	7	100
844	4	58 ± 9‡
17924	3	64 ± 18
ALG Lot		
162	3	74 ± 23
15343	3	41 ± 15
181S	3	50 ± 22
97412	7	74 ± 21
HIgG	3	18 ± 3

n, number of experiments.

*PBMNC were used instead of E-rosette purified T cells because previous studies have shown that E rosetting can stimulate T cells from aplastic anemia patients (reference 9) and normals (reference 37). In two experiments, cells surface phenotyping before and after stimulation showed that the percentage of T cells (Leu 4$^+$) increased from 79% to 86%, activated T cells (Leu 4$^+$, IL 2R$^+$) from 4% to 13%, while B cells (Leu 12$^+$) decreased from 9% to 7%.

†Mean ± SEM.

‡Relative blastogenesis values were obtained by normalizing the maximal SI of each ATG and ALG lot for each experiment with the SI of ATG lot 731 which was given an arbitrary value of 100. ATG lot 731 was included in each experiment. The stimulation index was 9.0 ± 2.4. for ATG lot 731 and 1.6 ± 0.3 for HIgG. The cpm of unstimulated cells and ATG lot 731-stimulated cells were 188 ± 48 and 2029 ± 365, respectively.

DISCUSSION

Both ATG and ALG precipitated a limited number of antigens from the cell surface membranes of thymocytes and lymphocytes. These bands almost certainly do not reflect all the antigens recognized by the horse sera, as the presence of certain major protein components will obscure minor bands during exposure of the autoradiograph. In addition, some of the densest bands precipitated most likely represent common cell membrane proteins, as ATG has been shown to recognize many normal human cell types[15] and their molecular weights do not correspond to those for known T-cell–specific proteins. Nonetheless, while the patterns of immunoprecipitation were roughly similar within ATG and ALG lots, differences in proteins precipitated by ATG compared to ALG were evident. These differences were also recognized in quantitative blocking experiments with monoclonal antibodies to T-cell antigens. Remarkable in these experiments was the much greater ability of ALG to block binding of at least two antigens, the pan-T cell antigen Leu 1 and HLA-DR. While there was greater variability among lots of ALG than among lots of ATG in this assay, even the least potent lot of ALG was 10 times more active in blocking binding to these antigens. That the blocking ability of ATG and ALG was due to direct binding rather than steric hindrance could be demonstrated in a case in which we were technically able to immunoprecipitate with monoclonal antibodies; in this instance, preclearing of lysates with ATG and ALG but not HIgG removed HLA-DR antigens (data not shown).

All the ATG and ALG preparations tested showed cytotoxic activity for T cells. Despite their poorer performance in some antibody competitions, all the ATG lots were equivalent to the best ALG lots in cytotoxicity. The ability to effect lysis must depend not only on the specific antigens recognized, but on factors such as the physical arrangement of antigens on the cell surface and the ability of antibodies to fix complement. There were no relative differences in cytotoxicity using rabbit or human serum as a source of complement. Furthermore, the concentrations of horse antisera needed for maximal cytotoxicity with human sera (160 to 320 ug/mL) were lower than the reported mean peak plasma levels of equine IgG after ATG infusion (727 ± 310 ug/mL).[30,31] It may be difficult to relate in vitro cytotoxicity experiments to clinical performance as immunosuppressive agents, as the correspondence might be, for example, to suboptimal as opposed to plateau concentrations measured in vitro or dependent on the regimen for administration of sera to patients. The in vitro data suggests that the in vivo properties of ATG and ALG may be concentration related; T cells may be stimulated at low concentations, whereas at higher concentrations, complement-mediated cytotoxicity and inhibition of T-cell proliferation by blocking important cell surface proteins such as the interleukin-2 receptor (CD25),[32,33] the E-rosette receptor (CD2),[34] or the T-cell receptor-associated antigen (CD3)[35,36] may predominate.

A clue to the mechanism of action of ATG and ALG might be provided by the comparative laboratory analysis of a lot (ALG 97412) described as being relatively ineffective in clinical trials in aplastic anemia patients.[17] Unfortunately, clinical data from Europe and the United States are contradictory in regard to the "activity" of this particular lot. In an American study (reviewed in 38), this lot led to a 31% rate of transfusion independence in 35 patients as opposed to a 69% response rate in a previous study of 29 patients by the same investigators using ALG EC162.[4] However, use of lot 97412 in Basel resulted in response of 9 of 12 patients with severe or moderate aplastic anemia, similar to excellent results obtained with a previous active lot (ALG 15343) and a subsequent lot (ALG 181S). While the reason for the discrepancy between the American and European experience with lot 97412 will probably never be determined, it is difficult to conclude that this ALG lot was clinically ineffective.

Our studies were undertaken on the report of clinical inactivity of lot 97412.[17] The results of our blocking experiments are similar to a published comparison of this "inactive" ALG lot and an active ALG lot (EC162)[38]; in particular, the relative amounts of antibodies specific for known T-cell antigens were similar in the two studies. While Smith et al[38] concluded that the overall 2- to 5-fold decrease in antibody blocking activity of the "inactive" lot, rather than the absence of a single antibody specificity, might account for clinical inactivity, we found the "inactive" lot very similar to another known active ALG lot (15343). The immunostimulatory capacity of this "inactive" lot, as determined by blastogenesis assays and hematopoietin production by T cells was equivalent to or superior to active lots of ALG and ATG. Although the "inactive" lot was less cytotoxic at suboptimal concentrations and failed to plateau even at concentrations 4- to 8-fold greater than the concentrations required for most other lots, this pattern was similar to that observed with an active lot, 181S. There was greater lot to lot variability among ALGs than among ATGs (which were remarkably homogenous in their properties as tested), but even when viewed independently of the clinical data, our laboratory studies failed to show a unique property of lot 97412 not shared by other ALG lots.

A question that has not yet been addressed in clinical trials is the relative effectiveness of ATG versus ALG. Response rates to ATG have averaged about 50%,[6] whereas the first American trial of ALG resulted in a response rate of 68%[4] and more recent Swiss data have suggested response rates as high as 85% with ALG in combination with adjuvant therapies.[5] If these response rates are truly different, they may reflect differences in patient selection, clinical management, the addition of high-dose corticosteroids and androgens, and the regimen for administration of the horse serum in addition to intrinsic differences in the preparations. Patients in Europe are often observed for several months for spontaneous recovery prior to therapy; the use of isolation and antibiotics is not uniform among centers; and androgens are employed with ALG more enthusiastically abroad[5] than in the United States.[19] In addition, there may be an advantage to administering horse serum in large doses over a short period of time (as in most ALG protocols), prior to the onset of serum sickness and more rapid clearance of horse IgG,[39] rather than over 10 to 28 days (as in most ATG protocols). If intrinsic properties of ALG are responsible for higher response rates in clinical trials, our results would direct attention to proteins such as Leu 1 and HLA-DR, which are much better bound by ALGs than ATGs.

Monoclonal antibody therapy of aplastic anemia has not been successful.[40,41] Another approach to more specific and less toxic immunotherapy of aplastic anemia would be the production of polyclonal antisera directed against a limited number of defined antigens present on target T cells. While the broad antibody specificities and multiple biologic properties of ATG and ALG have hindered the elucidation of their mechansim of action, comparison using these assays of ATGs and ALGs with more restricted antisera may ultimately be revealing.

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Glycoprotein V Is Not the Thrombin Activation Receptor on Human Blood Platelets

By Denise Bienz, Wolfgang Schnippering, and Kenneth J. Clemetson

Thrombin activation of platelets involves two receptors: glycoprotein Ib (GPIb), which affects the kinetics of the response; and, as a strong candidate for the second, essential receptor, GPV, a hydrophobic, 82-kd glycoprotein with an isoelectric point (pI) of pH 5.85 to 6.55. Whole platelets were treated with endogenous platelet calcium-activated proteases, yielding a major fragment, GPV$_s$, with molecular weight (mol wt) of 79 kilodaltons (kd). The fragment was purified by affinity chromatography on wheat germ agglutinin followed by ion exchange chromatography on DEAE-Sephacel using first a 0 to 0.7-mol/L and then a 0 to 0.3-mol/L NaCl gradient. A rabbit was immunized with the purified GPV$_s$ for preparation of polyclonal antibodies. Crossed immunoelectrophoresis and two-dimensional polyacrylamide gel electrophoresis (PAGE) electrophoretic blotting with the separate phases of a Triton X-114 phase partition of human platelets showed the characteristic pattern of GPV in the hydrophobic phase. During thrombin-induced platelet aggregation GPV is hydrolysed, releasing a fragment, GPV$_{fl}$, to the supernatant. The fragment GPV$_{fl}$ still contains a thrombin-binding site. Anti-GPV antibodies blocked GPV proteolysis, but did not inhibit platelet activation induced by thrombin. We conclude that proteolysis of GPV by thrombin is not essential for platelet activation.

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THROMBIN IS A POWERFUL physiological activator of platelets and other cells. Although the overall mechanism of activation is complex it seems certain that a proteolytic step is necessary since when the active site of thrombin is blocked it is no longer able to activate platelets.[1] The enzyme appears to act at the platelet surface without entering the cell.[2,3] A transmission system must therefore exist to carry the signal across the platelet membrane. There is evidence that the platelet membrane glycoproteins Ib and V (GPIb and GPV) have a role in thrombin activation of platelets, and it has been suggested that these glycoproteins may have receptor functions. GPIb contains a binding site for thrombin[4] which influences the kinetics of the platelet response,[5] but it is not the essential receptor for thrombin activation.[6]

GPV, a membrane-associated 82-kilodalton (kd) platelet glycoprotein, is hydrolyzed during thrombin activation to yield a 69.5-kd fragment, GPV$_{fl}$.[7,8] Hydrolysis is independent of platelet shape change, secretion, and aggregation, and is therefore directly caused by thrombin and is not a secondary event.[9] As such GPV has been regarded as a prime candidate for the thrombin activation receptor on platelets.[5,10]

On the other hand there is evidence against GPV as the essential thrombin receptor on platelets. Both GPIb and GPV are absent in platelets from patients with Bernard-Soulier syndrome[11,12,13] but the platelets can nevertheless be activated by thrombin even if an amount larger than normal is required.[14]

Platelet activation can also be inhibited under conditions where GPV hydrolysis is normal.[6] Most of GPV (plus several other glycoproteins) can be removed with chymotrypsin or endogenous platelet calcium-dependent proteases without affecting the extent of platelet activation, only the rate of activation is altered.[10] These results cast doubt on the role of GPV as the essential thrombin receptor on platelets but it might still participate in postreceptor signal processing.

To clarify the function of GPV in platelet activation by thrombin, we have purified GPV$_s$, a fragment of GPV produced by cleavage with platelet calcium-activated proteases, and produced antibodies against it. We have examined the influence of the antibodies bound to GPV on proteolysis of GPV and activation of the platelets. Our results lead to the conclusion that proteolysis of GPV by thrombin is not essential for platelet activation and that therefore GPV is not the thrombin activation receptor on human platelets.

MATERIALS AND METHODS

Isolation of human blood platelets. Platelets were isolated from citrate-treated blood collected for the Central Laboratory of the Swiss Red Cross, within 20 hours after collection.[15] The buffy coats were transferred into a buffered glucose solution to yield platelet-rich plasma containing about 20 mmol/L glucose, 12 mmol/L sodium phosphate buffer, and about 4 × 10^9 platelets per mL.[16] The platelets were isolated by centrifugation and were washed twice with 12 mmol/L sodium citrate, 30 mmol/L glucose, 120 mmol/L NaCl, and 10 mmol/L EDTA buffer, pH 6.5, and once with 10 mmol/L Tris/HCl, 150 mmol/L NaCl, and 10 mmol/L EDTA buffer, pH 7.4.

Isolation of glycoprotein V fragment (GPV$_s$). Washed platelets were suspended in 0.14 mol/L NaCl, 5 mmol/L $CaCl_2$, and 10 mmol/L Mes/NaOH, pH 6.5. One quarter of this suspension was cooled to 4 °C and sonicated with a B-30 sonifier (Branson Sonic Power, Danbury, Conn) for two minutes (output control, 7; 50% duty cycle, pulsed mode). The sonicated platelets as a crude source of calcium-activated proteases were added back to the rest of the platelet suspension which was left for 20 minutes at 37 °C. The calcium concentration was previously adjusted to 20 mmol/L with $CaCl_2$ solution. To prevent further degradation, phenylmethylsulfonyl fluoride (PMSF) in methanol and N-ethylmaleimide (both 2 mmol/L final concentration) were added and the suspension centrifuged at 100,000g for one hour. The supernatant, to which EDTA (5 mmol/L final concentration) was added, was applied to a wheat

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germ agglutinin–Sepharose 4B column (Medac, Hamburg, FRG) and the bound material eluted with 2.5% *N*-acetylglucosamine.[17] Bound GPV$_s$ was separated from glycocalicin by chromatography on DEAE-Sephacel (Pharmacia, Uppsala, Sweden) using a 0 to 0.7-mol/L NaCl gradient. Further purification was achieved by a second chromatography on DEAE-Sephacel using a 0 to 0.3-mol/L NaCl gradient.

Preparation of rabbit antibodies, IgG and F(ab')₂ fragments. A rabbit was immunized at two-week intervals for six weeks with GPV$_s$ (0.25 mg/mL) in physiological saline, mixed 1:1 with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich). After five weeks of rest, a booster immunization was given. The antiserum titer was regularly checked by Ouchterlony double diffusion in agarose and later by Laurell immunoelectrophoresis, and high-titer bleedings were pooled. IgG was prepared from the rabbit antisera by treatment[18] with DEAE-Sephacel.

F(ab')$_2$ fragments of rabbit IgG were prepared[19] by digestion with papain followed by ion exchange chromatography on CM-cellulose (Whatman CM52, W. and R. Balston, Kent, England) and gel filtration on Ultrogel AcA 34 (LKB-Producter AB, Bromma, Sweden).

Triton X-114 phase separation. Washed platelets were solubilized and separated into hydrophobic and hydrophilic phases by Triton X-114 (Sigma, St Louis) phase partition[20] as previously described.[21]

Polyacrylamide gel electrophoresis. Two-dimensional polyacrylamide gel electrophoresis (isoelectric focusing/5% to 15% gradient polyacrylamide gel electrophoresis) was performed as described earlier.[22] One-dimensional gel electrophoresis was done with 1.5-mm thick, 5% to 15% polyacrylamide gradient gels, using combs with 4.5-mm slots. The samples (10 to 50 μg) contained 1% sodium dodecyl sulfate (SDS) and 1% dithiothreitol for reducing conditions and were heated to 100 °C for two minutes before electrophoresis. The gels were silver-stained by the method of Morrissey[23] or were prepared for fluorography.[24]

Electrophoretic blotting. Glycoproteins were transferred from gels to nitrocellulose sheets (Schleicher and Schüll, Dassel, FRG) by the method of Towbin.[25] The binding to the blot of the primary rabbit antibody against GPV$_s$ was detected with peroxidase-labeled goat antirabbit IgG (Bio-Science Products AG, Emmenbrücke, Switzerland) using 4-chloro-1-naphthol (Sigma) as substrate.

Affinity chromatography on thrombin-Sepharose 4B. 1 mg bovine thrombin (1,000 U/mL, Calbiochem, Zürich, Switzerland) was coupled to Sepharose 4B by the cyanogen bromide method.[26] GPV$_s$ purified as described above was loaded on the thrombin column and washed with 20 mmol/L Tris/HCl, pH 7.4 to remove any excess of GPV$_s$ over the capacity of the column. The bound material was eluted with heparin (40 U/mL; Sigma) in Tris buffer.

Surface labeling by the periodate/[^{3}H]-NaBH$_4$ method. Platelets were isolated from platelet-rich plasma, washed and surface-labeled by the periodate/[^{3}H]-NaBH$_4$ method[27] as modified by Steiner.[28]

Thrombin treatment. Washed platelets (5×10^9/mL) were ^{3}H-labeled and preincubated with anti-GPV antibody (antiserum), preimmune serum, or platelet buffer (as control) for ten minutes. Thrombin (8 U/mL; Hoffmann-La Roche, Basle, Switzerland) was added and the suspension left for three minutes at 37 °C. Then the platelets were centrifuged at 1100g for ten minutes. In one half of the supernatant, proteolysis was inhibited with *p*-amidinophenylmethylsulphonyl fluoride (APMSF, 1 mmol/L final concentration; Calbiochem, Zürich, Switzerland) and leupeptin (100 μg/mL final concentration; Fluka AG, Buchs, Switzerland). The other half was treated again with thrombin (400 U/mL) for 30 minutes at 37 °C and then proteolysis was inhibited with APMSF and leupeptin. The thrombin-treated platelets were washed twice with platelet buffer and solubilized in 1% Triton X-114, 10 mmol/L Tris/HCl, 154 mmol/L NaCl, 20 mmol/L EDTA, 2 mmol/L PMSF, 100 μg/mL leupeptin for 30 minutes at 0 °C. The solution was then centrifuged for 30 minutes at 100,000g at 0 °C and the supernatant used for one-dimensional gel electrophoresis.

Aggregation studies. Platelet-rich plasma was washed twice in 5 mmol/L glucose, 3 mmol/L KCl, 3 mmol/L Mes (2-(*N*-morpholino) ethanesulfonic acid), pH 6.5 and suspended at a concentration of 3×10^8/mL in the same buffer. Aliquots of 400 μL in aggregometer tubes were incubated either with anti-GPV antibody [F(ab')$_2$ fragments] or with buffer as control before treatment with thrombin (0.05 U/mL), ristocetin (1.5 mg/mL, Lundbeck, Copenhagen, Denmark), human von Willebrand factor (100 μL plasma), adenosine diphosphate (ADP) (1.2 μg/mL, Serva, Heidelberg, FRG) or collagen (5 μg/mL, Hormon Chemie GmbH, Munich, FRG).

RESULTS

Isolation of GPV fragment. During earlier work on the isolation of platelet glycocalicin[5] it was noted that during treatment of platelets with endogenous calcium-activated proteases a large fragment of GPV was released from the platelet as well as glycocalicin. The fragment, referred to here as GPV$_s$, contains the thrombin cleavage site and coisolates with glycocalicin on affinity chromatography on wheat germ agglutinin. This provides a rapid isolation step from the platelet supernatant and as shown in Fig 1, GPV$_s$, due to its more basic pI, can be separated from glycocalicin by ion exchange chromatography on a DEAE-Sephacel column with a 0 to 0.3-mol/L NaCl gradient. The pooled fractions after this step contained only GPV$_s$ when examined

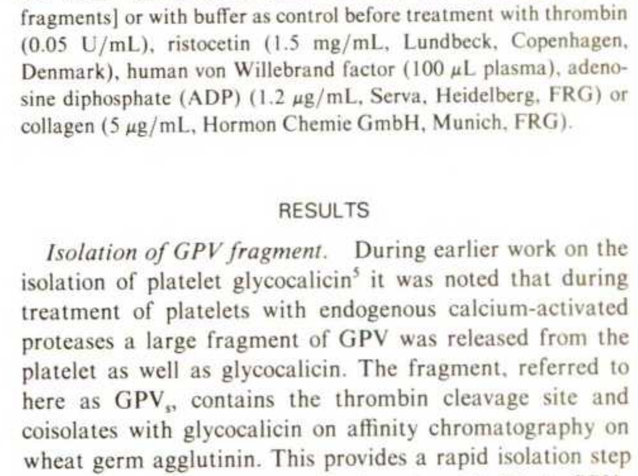

Fig 1. (A) Elution diagram of the DEAE-Sephacel column (eluted with a 0 to 0.3-mol/L NaCl gradient), the last step in GPV$_s$ purification. Each fraction was analysed by SDS-polyacrylamide gel electrophoresis (B) and the proteins were silver-stained by the method of Morrissey.[23] Lane a, starting material for the DEAE-Sephacel column; lanes b through f, Fractions 15 through 19, containing GPV$_s$.

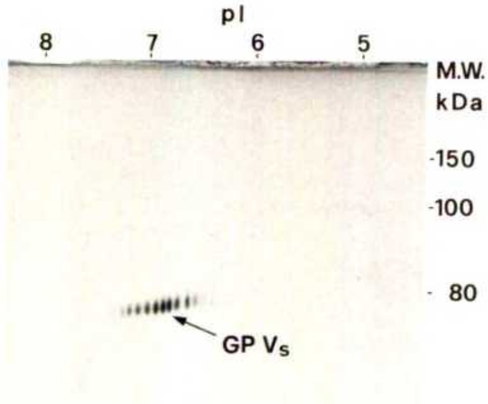

Fig 2. Two-dimensional polyacrylamide gel (isoelectric focusing/gel electrophoresis) of the isolated GPV$_s$, the fragment obtained by calcium-activated protease treatment. The gel was silver-stained by the method of Morrissey.[23]

by two-dimensional gel electrophoresis followed by silver staining (Fig 2). The mol wt and the heterogenous pI found for GPV$_s$ were similar to those reported earlier for GPV.[29] As previously reported[7,8] GPV was the only surface glycoprotein on platelets to be cleaved by thrombin. The cleavage fragment GPV$_{fl}$ from intact platelets was identical to that obtained by treating GPV$_s$ with thrombin.

Preparation of antibody. The purified GPV$_s$ was used to immunize a rabbit for preparation of polyclonal antibodies. The specificity of the antiserum was determined by immunoblotting with two-dimensional polyacrylamide gel separations of platelet fractions. Figure 3 shows such an immunoblot of the Triton X-114 phase from a phase partition of platelets with the anti-GPV$_s$ serum. GPV was the only component that was stained. The Triton X-114 phase was used because it is enriched in most platelet membrane glycoproteins including GPV[21] (Fig 4). Immunoblots with whole platelet preparations also showed that GPV$_s$ was the only component to react with the anti-GPV$_s$ serum although the staining was relatively weaker. The aqueous phase of the Triton X-114 phase separation did not contain any components which reacted with the anti-GPV$_s$ serum (data not shown) but contained all the GPIb (Fig 4). On one-

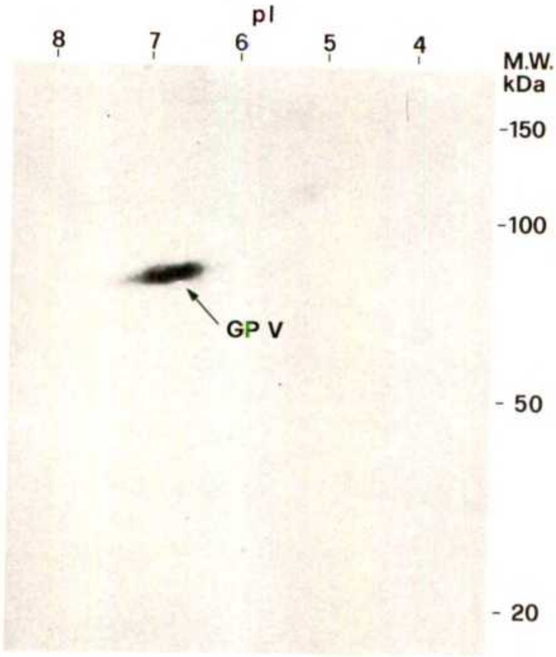

Fig 3. Immunoblotting of a two-dimensional polyacrylamide gel (isoelectric focusing/gel electrophoresis) separation of the Triton X-114 phase from phase partition of platelet proteins with anti-GPV$_s$ antibody as first antibody. Faint staining in the higher molecular weight, more acidic region is nonspecific background.

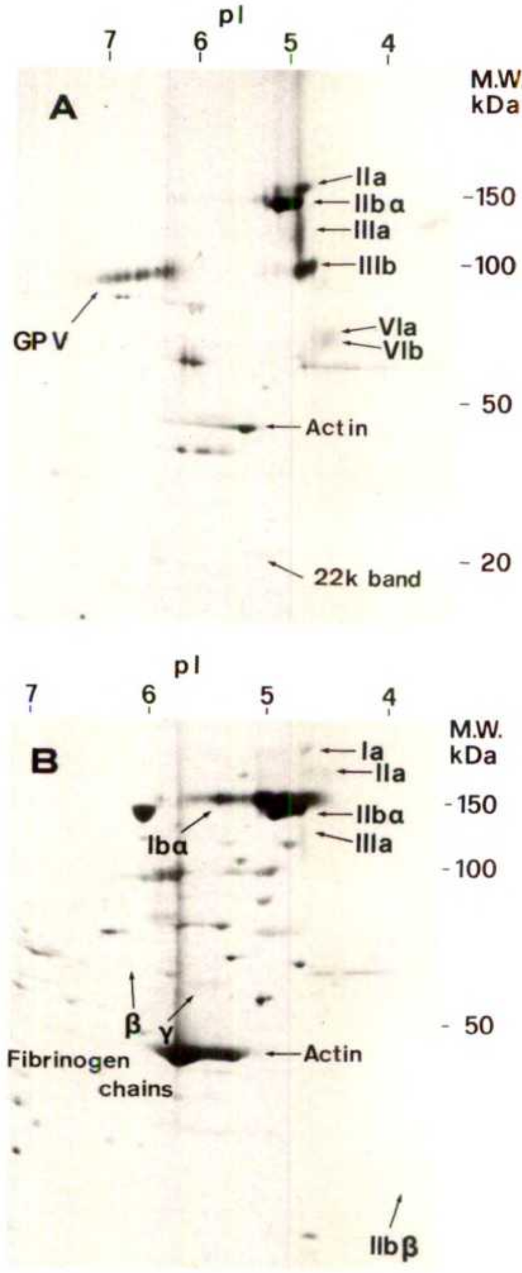

Fig 4. Two-dimensional gel electrophoresis (isoelectric focusing/gel electrophoresis) separation of platelet proteins, silver-stained by the method of Morrissey.[23] (A) Triton X-114 phase from phase partition of platelet proteins; (B) aqueous phase from phase partition of platelet proteins.

dimensional gels the component recognized by the anti-GPV$_s$ serum showed the characteristic behavior of GPV and moved to a lower mol wt of 69,500 d in the supernatant of platelets treated with thrombin (data not shown).

Influence of the antibody on GPV cleavage by thrombin. Since GPV is so far the only substrate for thrombin detected on the platelet surface, the effect of anti-GPV$_s$ IgG on the cleavage of GPV from platelets and on the activation of platelets by thrombin were studied. Cleavage of GPV from periodate/[^{3}H]NaBH$_4$ labeled platelets by thrombin treatment gives rise to the readily identifiable fragment GPV$_{f1}$ in the supernatant[7,8] which provides a semiquantitative measurement of the extent of the cleavage reaction.[30]

When surface-labeled platelets were treated with anti-GPV$_s$ IgG before exposure to thrombin, cleavage of platelet GPV to GPV$_{f1}$ was strongly inhibited in comparison to platelets treated with preimmune serum (Fig 5). To demonstrate that the GPV fragment in the supernatant really is GPV$_{f1}$ and not GPV$_s$, the cleavage product with calcium-activated proteases, the supernatant was treated again with a large excess of thrombin. No further degradation of the GPV fragment was detectable (Fig 5C). The GPV$_{f1}$ band in Fig 5C, lanes c and d, appears to run slightly lower because of the presence of an unlabeled protein band from the thrombin preparation running just above. This result indicates that the antibodies block thrombin binding to the cleavage site. The anti-GPV$_s$ IgG also blocked cleavage of GPV$_s$ to GPV$_{f1}$ in the supernatant from labeled platelets, in the presence of a large excess of thrombin, confirming that the inhibition was due to binding to GPV and not to an indirect effect on the platelets (data not shown).

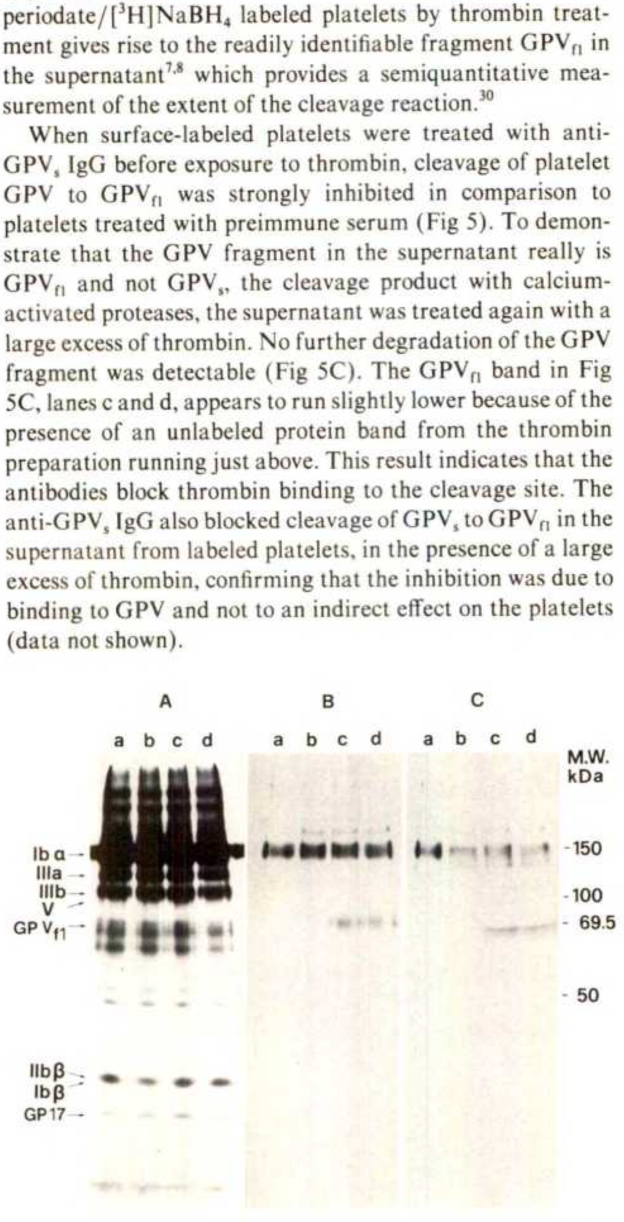

Fig 5. Fluorograms of SDS-polyacrylamide gel electrophoretic separation of platelet membrane glycoproteins labeled by the periodate/[^{3}H]-NaBH$_4$-method. Washed platelets were treated as followed: (a) only with anti-GPV$_s$antibody; (b) first with anti-GPV$_s$ antibody and then with thrombin; (c) with preimmune serum and then with thrombin; (d) only with thrombin. After thrombin treatment the pellet suspension was centrifuged, to give a platelet pellet (A) and GPV$_{f1}$ cleaved by thrombin in the supernatant (B). Part of the supernatants were tested again with a large excess of thrombin (C). The position of GPV$_{f1}$, present only in the supernatants, is indicated on the left of the figure.

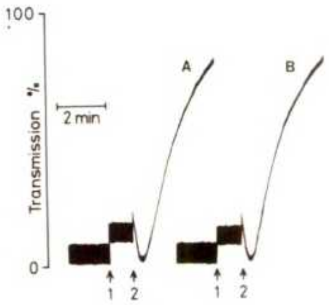

Fig 6. Effect of anti-GPV$_s$antibody on aggregation of thrombin activated platelets. (A) Control: Platelets (400 μL; 3 × 10^8/mL) were pretreated (1) with buffer (20 μL) and stimulated (2) with thrombin (20 μL; 0.05 U/mL). (B) Platelets were pretreated (1) with (Fab')$_2$ fragments of anti-GPV$_s$ antibodies (20 μL) and stimulated (2) with thrombin (20 μL; 0.05 U/mL).

Platelets treated with F(ab')$_2$ fragments of anti-GPV$_s$ IgG were compared with untreated and with preimmune serum–treated platelets in aggregation with thrombin to see if the activation of platelets by thrombin is influenced when cleavage of GPV is blocked (Fig 6). Binding of F(ab')$_2$ fragments of anti-GPV$_s$ IgG had no effect on the platelet aggregation response to thrombin, ADP, collagen, or ristocetin/von Willebrand factor, nor did it delay the shape change response.

Binding of GPV$_s$ to insolubilized thrombin. Binding and cleavage of GPV$_s$ were studied using thrombin which was coupled to Sepharose 4B beads. A solution of GPV$_s$ was loaded on the column and eluted with buffer. Any excess of GPV$_s$ over the capacity of the column appeared in the flow-through (Fig 7, lane a) but the bulk of the GPV$_s$ bound to the thrombin. Attempted elution with a variety of buffers was unsuccessful but heparin gave an elution peak. Examination of this peak by one-dimensional SDS–polyacrylamide

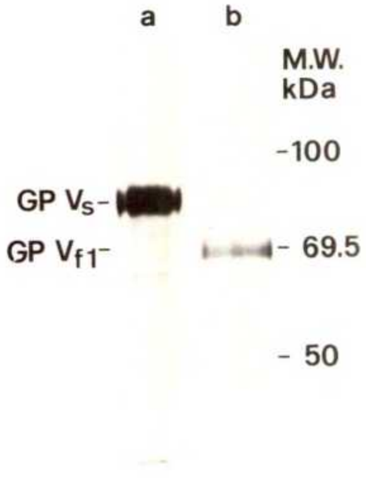

Fig 7. One-dimensional gel electrophoresis of two fractions from a thrombin–Sepharose 4B column; (a) Excess of GPV$_s$, over the capacity of the column recovered in the flow-through. (b) Bound material eluted with heparin. Proteins were silver-stained by the method of Morrissey.[23]

gel electrophoresis showed a band at the same mol wt as the GPV_{fl} fragment (Fig 7, lane b). A smaller piece corresponding to the difference between GPV_s and GPV_{fl} was not detected in the flow-through or the eluate.

DISCUSSION

GPV is the only demonstrable substrate for thrombin on human platelets and for this reason has long been a prime candidate for the platelet thrombin receptor. However, in recent years several studies have cast doubt on the role of GPV in the activation of platelets by thrombin.[9,10,30,31] The development of a simplified method for the purification of a major proteolytic fragment of GPV has provided the opportunity to prepare antibodies to GPV and to test their effects on platelet activation by thrombin. The preparation of a major proteolytic water-soluble fragment of GPV is based on treatment of platelets with a crude preparation containing calcium-activated proteases. The calcium-activated proteases remove not only glycocalicin from GPIb but also most of GPV as the main membrane glycoprotein fragments found in the supernatant. This fragment of GPV, GPV_s, is probably what was originally isolated and described as GPV[29] since it has similar properties. We have already shown[5,21] that the intact GPV molecule is slightly larger and hydrophobic. Here we demonstrate again that intact GPV is entirely found in the hydrophobic phase of a Triton X-114 phase partition. Antibodies prepared against GPV_swere shown to be specific for platelet GPV by immunoblotting. Since GPV_s contains the thrombin cleavage site and can be cleaved to GPV_{fl} by thrombin, we examined the effect of these antibodies on thrombin proteolysis of membrane-bound intact GPV. As thrombin treatment causes platelet activation and the release of several proteins from storage granules it is easier to observe the effect on GPV by using surface-labeled platelets. Platelets that were pretreated with anti-GPV_s antibodies before adding thrombin showed a greatly reduced cleavage of GPV to GPV_{fl}. Even a large excess of thrombin (8 U/mL) caused no detectable release of GPV_{fl}, indicating that the antibodies had blocked the cleavage site. However, in aggregation studies, pretreatment of platelets with anti-GPV_s antibodies had no effect on platelet activation by thrombin. As little as 0.05 U/mL thrombin (160-fold less than in the GPV_{fl} release study) caused normal aggregation. These findings and the dose-response and time-response relationship studies of McGowan et al[10] indicate that the hydrolysis of GPV by thrombin is not essential for platelet activation and that GPV is therefore not the thrombin receptor on platelets. This result supports earlier work with Bernard-Soulier syndrome platelets where GPV as well as GPIb and GP17 (GPIX) are absent[11-13] but the platelets even so respond to thrombin.[14] They also agree with the results of McGowan et al[10] and White and Knupp[30] who found no direct relationship between cleavage of GPV by thrombin and the extent of the platelet response. Since GPIb, although a thrombin receptor, only influences the rate of response to thrombin but not the extent and is also not necessary for platelet activation, the essential platelet component must be a thrombin-sensitive surface protein that has not yet been identified. Because surface labeling methods have so far been unsuccessful in finding a suitable candidate, it must be concluded either that the receptor is poorly labeled if at all or that only a very minor portion is cleaved during platelet activation.

The role of GPV in platelet function and in relation to thrombin remains obscure. Antibodies to GPV_s did not affect platelet activation by several stimulators including thrombin. However, the fact that GPV is cleaved by thrombin may imply that it has some role in the physiological activity of thrombin other than in activation of platelets. The lack of any apparent relationship between GPIb and GPV either through complex formation such as is found between GPIb and GP17 (GPIX)[32,33] or through immunological cross-reactivity makes the absence of both glycoproteins in the recessive genetic disorder Bernard-Soulier syndrome even more puzzling. As we show here and in previous work,[21] GPIb and GPV separate in different phases in Triton X-114 phase partition. Immunoprecipitation experiments with anti-GPIb and anti-GPV antibodies (data not shown) also failed to show coprecipitation of these glycoproteins. Immunoblotting experiments showed no cross-reactivity between GPIb and GPV (Fig 3). One possibility that remains to be explored is that both glycoproteins have similar glycosylation steps during synthesis and that the defect in Bernard-Soulier syndrome lies there. A solution to this problem will require the characterization of the genes for these glycoproteins in normal and in Bernard-Soulier syndrome patients.

We have investigated the binding of GPV_s to insolubilized thrombin and found that it bound strongly. On elution with heparin it was the cleavage fragment GPV_{fl} that was recovered. This shows that GPV_{fl} is still capable of binding thrombin and, when thrombin cleaves GPV, the thrombin remains associated with the soluble GPV_{fl} that is removed from the platelet. The presence of GPV_{fl} in plasma may be a good indicator of platelet exposure to thrombin under in vivo conditions and is under investigation. The availability of a specific antiserum to GPV should allow studies on its tissue distribution and on its suitability as a marker during megakaryocyte development.

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Estimates of Iron Sufficiency in the US Population

By James D. Cook, Barry S. Skikne, Sean R. Lynch, and Molly E. Reusser

Traditionally the iron status of a population is assessed by estimating the prevalence of iron deficiency anemia. This approach is inadequate in countries where the diet is heavily fortified with iron because it conveys no information about the iron-replete segment of the population. In the present study iron status of a US adult population was evaluated using data collected in the second National Health and Nutrition Examination Survey (NHANES II). Body iron was estimated in each of 2,829 individuals from measurements of hemoglobin concentration, serum ferritin, transferrin saturation, and erythrocyte protoporphyrin. When individuals between 18 and 64 years of age were divided on the basis of sex and menstrual status, body iron reserves were normally distributed and averaged 309 mg in women 18 to 44 years, 608 mg in women 45 to 64 years, and 776 mg in men 18 to 64 years. The dispersion of storage iron in these groups was similar, with standard deviations of 346, 372, and 313 mg, respectively. The prevalence of iron deficiency anemia was surprisingly low, ranging from only 0.2% in adult men to 2.6% and 1.9% in pre- and postmenopausal women, respectively. Epidemiologic methods that examine iron status in the entire population assume importance in light of evidence that in certain segments of the US population, iron deficiency anemia is now less common than the homozygous state for hereditary hemochromatosis.

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THE IRON STATUS of a population is presently assessed by determining the proportion of individuals with laboratory values outside a predefined cutoff level. This approach is flawed by the extensive overlap in laboratory values between normal and iron-deficient individuals. With hemoglobin measurements, for example, the number of normal individuals with values below the accepted cutoff level commonly exceeds the number with true iron deficiency anemia even when the prevalence of iron deficiency is high.[1,2] A more important limitation with current epidemiologic methods is that they reflect only the prevalence of iron deficiency and ignore the iron-replete segment of the population. This is a concern in the United States, where iron is liberally consumed in supplements and fortified foods and where the heterozygote frequency for hemochromatosis is now estimated to exceed 10%.[3]

Several iron measurements are available for detecting varying degrees of iron lack.[4] In clinical practice these laboratory indices are commonly integrated to estimate body iron stores in an individual patient. In the present study this principle was used to estimate body iron in a random sample of the US population. The data base consisted of 2,829 adult subjects examined as part of the second National Health and Nutrition Examination Survey (NHANES II). The results indicate a low prevalence of iron deficiency and a broad distribution of body iron reserves.

MATERIALS AND METHODS

NHANES II. This survey was based on a stratified probability cluster sample of households from 64 separate geographic locations throughout the United States.[5,6] A total of 27,803 subjects aged 6 months to 72 years were identified of whom 20,322 were examined over a four-year period beginning in early 1976. A weighting procedure based on selection probabilities, adjustments for nonresponse, and poststratification adjustments was applied to each observation.[7] Each person was then considered to represent a certain number of individuals in the total US population. The data used for the present analysis were kindly provided by Cliff Johnson, National Center for Health Statistics. Estimates of the prevalence of iron deficiency anemia based on NHANES II have been published recently.[5,8]

Subsample selection. Because of the central importance of serum ferritin levels in estimating iron status, the analysis was limited to individuals in whom this measurement was available. Of the original 27,803 subjects sampled in NHANES II, serum ferritin measurements were performed in 5,157. Approximately one third of these were selected because of an abnormal red cell count, hemoglobin, hematocrit, mean corpuscular volume, or white cell count.[5] The objective was to include all persons with laboratory data suggestive of iron deficiency anemia. The remainder were selected randomly from the hematologically normal NHANES II population. Although the combined sample included a disproportionately high number of anemic individuals, the results can be extrapolated to the population by applying the weighting procedure.

To obtain relatively homogeneous subsets with regard to iron status, the present study was limited to adults between the ages of 18 and 65 years. Blacks were excluded to eliminate the effect of racial differences in hemoglobin concentration.[9,10] No persons were excluded because of abnormal laboratory values. Males between 18 and 64 years of age were analyzed as a single group (1,335), whereas because of the effect of menopause on iron status, women were divided into those below 45 years of age (937) and those 45 and above (557).

Laboratory measurements. Iron status was estimated from serum ferritin, erythrocyte protoporphyrin, transferrin saturation, and blood hemoglobin levels.[5,11] Erythrocyte protoporphyrin was analyzed fluorometrically in 10 μL whole blood using a modification of the extraction method of Sassa et al.[12] The results were converted to μg/dL packed red blood cells using the hematocrit determination. Serum iron was measured by the automated Technicon AA11-25 method and total iron-binding capacity (TIBC) was measured by the same technique after removing excess saturating iron with magnesium carbonate. Transferrin saturation was calculated by expressing the serum iron as a percent of the TIBC.

Serum ferritin determinations were performed at the University of Kansas Medical Center using a two-site immunoradiometric assay.[13] Ferritin protein was standardized against bovine serum albumin (BSA) using the Lowry technique.[14] Standards from a

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single batch of recrystallized human ferritin were diluted to 1,000 μg/L in buffered 5% BSA and stored at −20 °C. All assays were performed in triplicate at an initial dilution of 1:20. Samples with values <20 μg/L or >200 μg/L were reassayed at dilutions of 1:10 or 1:100, respectively, with diluted standards containing equivalent concentrations of normal rabbit serum. Quality control over the five-year span of the project was achieved by performing measurements at the beginning, midpoint, and end of each assay on an aliquotted pool of normal serum stored at −70 °C. At the conclusion of the survey ferritin measurements in each assay were adjusted to the overall average for this quality control.

Criteria of iron deficiency. Iron deficiency was defined using multiple criteria[5,15] as abnormal values for at least two of the following iron parameters: serum ferritin, transferrin saturation, and erythrocyte protoporphyrin. Serum iron was used in place of transferrin saturation in approximately 10% of sampled individuals in whom TIBC values were not available. Cutoff levels for these measurements are listed in Table 1. Iron deficiency anemia was considered present when, in addition, the hemoglobin concentration fell below the listed cutoff levels (Table 1).

Estimates of body iron. For this purpose body iron is divided into a storage compartment and a functional compartment comprising circulating hemoglobin and all nonstorage tissues. Body iron is expressed in relation to the storage compartment.[16] A positive value represents the amount of iron that could be removed from the body without inducing a deficit in the functional compartment. A negative value denotes iron deficiency and represents the amount of iron that must be returned to the body before iron stores can accumulate.

To calculate iron stores, individuals were divided into three levels of iron status on the basis of the initial laboratory measurements. In those with iron deficiency anemia (abnormal hemoglobin and at least two abnormal iron parameters), iron stores were calculated as follows:

$$\text{iron stores (mg)} = -15 \times (\text{mean HB} - \text{observed HB}) \qquad [A]$$

where mean hemoglobin (HB) is 140 g/L in women and 150 g/L in men. This calculation is based on the assumption that the deficit in body iron with anemia is inversely proportional to the hemoglobin concentration, that the mean hemoglobin concentration in normal men and women is 20 g/L higher than the cutoff level for anemia, and that 10 g/L circulating hemoglobin corresponds to 150 mg body iron. This algorithm was used in 8.5% of premenopausal women and 3.7% of postmenopausal women.

The formula used to calculate iron stores in those without iron deficiency anemia depended on the serum ferritin level. When this was less than 12 ug/L, serum ferritin, transferrin saturation, and erythrocyte protoporphyrin were weighted about equally in estimating the storage iron deficit. An index with values ranging from 0 to 5 was used as follows:

$$\text{iron stores (mg)} = -80 \times \text{index} \qquad [B]$$

To calculate this index a value of 1 was given for each of the following: serum ferritin below 9 μg/L, transferrin saturation below 16%, transferrin saturation below 10%, erythrocyte protoporphyrin above 70 μg/dL RBC, and erythrocyte protoporphyrin above 100 μg/dL RBC.

In the remaining individuals with normal serum ferritin levels, iron stores were calculated as follows:

$$\text{iron stores (mg)} = 400 \times (\log \text{SF} - \log 12) \qquad [C]$$

where log is the natural logarithm and SF is serum ferritin in μg/L. This formula is derived from quantitative phlebotomy studies indicating that 1 μg serum ferritin is equivalent to approximately 8 to 10 mg storage iron.[17-19] A log transformation was employed because of evidence that the removal of storage iron by phlebotomy in normal subjects with high iron stores produces a greater fall in the serum ferritin level than in those with low stores.[20] In subjects with borderline iron deficiency (serum ferritin 12 to 30 μg/L), algorithms B and C were both applied. Formula B was not used at higher serum ferritin levels because a low transferrin saturation or elevated erythrocyte protoporphyrin value was considered in this situation to more likely reflect chronic inflammation rather than iron deficiency.[21] Algorithm B was used alone in 17.2% of premenopausal women and 5.3% of postmenopausal women, and was used in conjunction with algorithm C in a further 7.5% and 4.0% of these groups, respectively. Formula C was used exclusively in more than 95% of men.

Table 1. Criteria of Iron Deficiency

	Abnormal Value
Serum ferritin (μg/L)	<12
Transferrin saturation (%)	<16
Serum iron* (μg/dl)	<50
Erthrocyte protoporphyrin (μg/dL RBC)	>70
Hemoglobin concentration (g/L)	
Men	<130
Women	<120

*Used only when transferrin saturation was not available.

RESULTS

Measurements of iron status in the three study groups are shown in Table 2. Values for erythrocyte protoporphyrin and transferrin saturation were similar in all groups, whereas serum ferritin levels differed markedly. The median ferritin of 27 μg/L in premenopausal women increased sharply to 63 μg/L in women over 45 years of age, but remained lower than the median of 92 μg/L observed in men. These values agree well with previous surveys in North America. For example, median values of 25 μg/L in premenopausal women and 94 μg/L in men were reported in the United States,[15] and geometric means of 23 μg/L and 93 μg/L, respectively, in a Canadian study.[22]

As anticipated, there was a striking difference in body iron in the three groups with mean values of 309 mg in premenopausal women, 608 mg in postmenopausal women, and 776 mg in men. The differences were even more dramatic at the lower end of the frequency distribution curve as reflected by fifth percentile values of −195 mg, −80 mg, and 242 mg iron, respectively. On the other hand, the upper end of the frequency distribution in postmenopausal women approached that of men with differences of less than 100 mg at the 90th and 95th percentiles. Interestingly, the width of the frequency distribution as reflected in the standard deviation was similar in the three groups with values between 313 mg, and 372 mg.

The cumulative frequency distribution of body iron approached a Gaussian distribution (Fig 1). In menstruating women a slight deviation from linearity occurred from 0 mg to 200 mg, whereas in postmenopausal women the points were slightly curvilinear, implying a mixture of two or more discrete populations. This became more apparent when the frequency distributions were plotted as histograms (Fig 2). Whereas the distribution in premenopausal women was

Table 2. Measurements of Iron Status and Estimated Iron Stores In Adults 18 to 64 Years

Measurement*	N	Mean	SD	Percentile						
				5	10	20	50	80	90	95
Female, 18 to 44 yr										
HB (g/L)	937	133	10	117	121	126	133	142	146	149
SF (μg/L)	937	37(24)†	38	3	7	11	27	57	82	108
EP (μg/dl)	937	55	20	37	40	43	52	64	74	83
TS (%)	860	27	12	11	14	18	25	37	42	47
ST (mg)	937	309	346	−195	−115	−73	324	623	770	879
Female, 45 to 64 yr										
HB	557	137	11	119	124	128	136	146	152	156
SF	557	78(56)†	68	11	18	27	63	116	152	193
EP	557	56	24	34	38	42	51	65	75	89
TS	503	26	9	14	15	19	25	32	36	43
ST	557	608	372	−80	108	324	663	907	1016	1110
Male, 18 to 64 yr										
HB	1335	152	11	135	139	143	152	161	166	170
SF	1335	113(85)†	94	22	32	46	92	157	207	299
EP	1335	50	27	34	37	40	46	56	63	70
TS	1335	30	10	16	18	21	28	37	43	47
ST	1335	776	313	242	370	537	801	1026	1135	1264

*HB, hemoglobin; SF, serum ferritin; EP, erythrocyte protoporphyrin; TS, transferrin saturation; ST, iron stores.

†Geometric means given in parentheses.

roughly symmetrical, it was negatively skewed in postmenopausal women. Because iron stores are known to expand following menopause, postmenopausal women were separated into smaller age groups (Fig 3). In women 45 to 49 years of age the distribution of iron stores was quite broad, whereas in the 50- to 54-year age range the distribution was bimodal. In women between 55 and 59 years the histogram approached a normal distribution, indicating that nearly a decade is required following menopause to achieve a homogeneous population in regard to iron status.

Various methods for estimating the prevalence of iron deficiency are compared in Table 3. The prevalence of iron deficiency anemia is appreciably higher when using a single criterion (abnormal hemoglobin) than when based on either multiple criteria or iron stores less than −300 mg, the latter two agreeing well. For example, in premenopausal women the estimated prevalence by these three techniques is 7.5%, 2.3%, and 2.6%, respectively. It should be noted that prevalence estimates expressed as a percentage in this table are lower than when calculated from the listed number of subjects because of the weighting procedure. For example, 60 of 937 or 6.4% of women between 18 and 44 years had iron stores below −300 mg as compared to a prevalence after weighting of 2.6%.

Similar comparisons were made for the prevalence of iron deficiency. Using a single criterion in younger women, the prevalence was substantially higher when based on serum

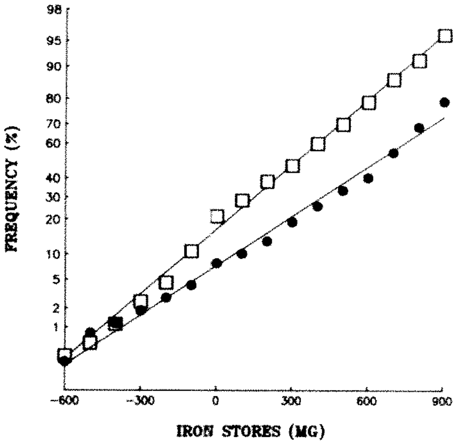

Fig 1. Cumulative frequency distributions of iron stores in women 18 to 44 (□) and 45 to 64 (●) years of age.

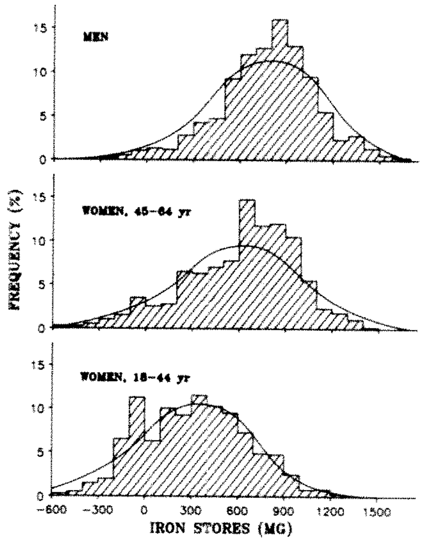

Fig 2. Histograms of the frequency distribution of body iron stores in adult men and women.

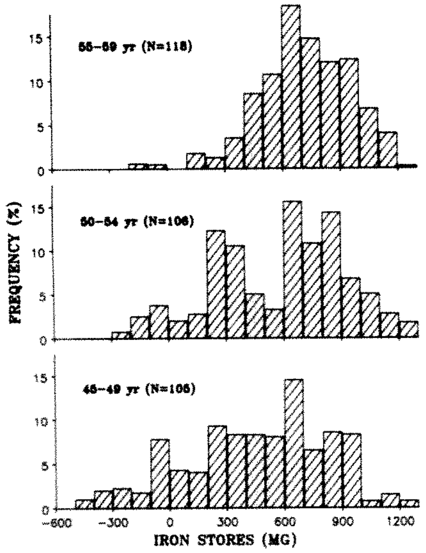

Fig 3. Frequency distributions of body iron stores in postmenopausal women.

ferritin levels (21.3%) than when based on transferrin saturation (14.5%) or erythrocyte protoporphyrin (12.1%). This difference is not unexpected because storage iron depletion as reflected by serum ferritin occurs prior to the onset of iron-deficient erythropoiesis as measured by transferrin saturation or erythrocyte protoporphyrin. It is interesting, however, that this pattern was reversed in both postmenopausal women and in men in whom the prevalence of mild iron deficiency was distinctly lower when determined by serum ferritin levels. This supports the suggestion that other disorders such as chronic inflammation or infection contribute to abnormal transferrin saturation or erythrocyte protoporphyrin levels in these population segments.[8]

When iron deficiency was defined by multiple criteria, the prevalence fell to 10% in premenopausal women and to only 4.1% and 0.7% in postmenopausal women and men, respectively. These values agree closely with the prevalence of iron deficiency defined as iron stores less than −100 mg; prevalence levels of 11.1%, 4.0%, and 0.8%, respectively, were observed. Interestingly, the prevalence increases almost twofold (22.3%, 7.6%, and 1.8%) if a cutoff level of 0 mg rather than −100 mg iron stores is used. Taken together these results indicate that the calculation of iron stores can be used to estimate the prevalence of iron deficiency with or without anemia, as well as to characterize the iron status of the entire population.

Table 3. Prevalence of Iron Deficiency in Adults Living in the US Measured by Various Criteria

	Women 18 to 44 yr		Women 45 to 64 yr		Men 18 to 64 yr	
	N	%	N	%	N	%
Iron Deficiency Anemia						
Single criterion	151	7.5	73	5.2	113	2.2
Multiple criteria	55	2.3	21	1.4	14	0.2
Storage estimate	60	2.6	24	1.9	16	0.2
Iron Deficiency						
Single Criterion*						
SF	213	21.3	45	5.9	38	1.6
TS	171	14.5	79	10.8	105	5.0
EP	147	12.1	92	12.3	75	4.2
Multiple Criteria	124	10.0	40	4.1	27	0.7
Storage Estimate						
Below 0 mg	226	22.3	56	7.6	45	1.8
Below −100 mg	129	11.1	38	4.0	30	0.8

*SF, serum ferritin; TS, transferrin saturation; EP, erythrocyte protoporphyrin.

One possible advantage of estimating iron stores in each individual is to improve the precision and reliability of population surveys. To evaluate this, groups of 50 women were selected randomly by computer from the total sample of 937 premenopausal women. In each of these samples the prevalence of iron deficiency anemia was estimated using a single criterion (abnormal hemoglobin), multiple criteria, or body iron stores less than −300 mg. The latter was determined by calculating the mean and standard deviation for body iron in each sample and determining the proportion of the population with iron stores less than −300 mg from tables of the normal distribution. When 10 such samples were drawn the prevalence based only on hemoglobin ranged widely from 14% to 42% (Fig 4). The estimated prevalence averaged only 4.5% when using multiple criteria with a range of 0% to 8%. The most consistent estimate of prevalence was obtained by calculating iron stores, with values ranging from only 3.3% to 7.5%. Weighting factors were not applied in this study.

DISCUSSION

Three separate computations were used to calculate body iron in the present report depending on whether laboratory measurements indicated iron deficiency anemia, iron deficiency, or residual iron stores. The calculation in anemic individuals (formula A) is relatively straightforward because the deficit in body iron is clearly related to hemoglobin concentration once the level has fallen below the cutoff level for anemia. This calculation might be further refined by using a formula based on body weight rather than assuming a

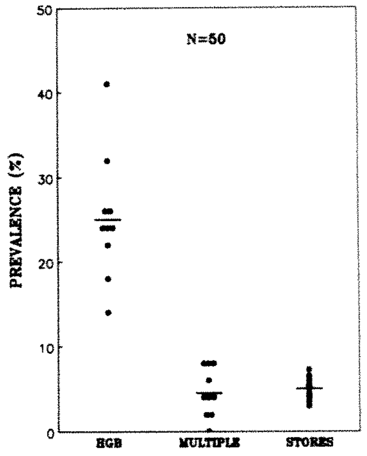

Fig 4. Comparison of methods for estimating the prevalence of iron deficiency anemia in 10 groups of 50 premenopausal women selected randomly from the NHANES II sample.

fixed value of 15 mg iron for each 1 g/L difference in circulating hemoglobin. Expression on a body weight basis will obviously be required if this approach is used in infants and children and may be useful when comparing iron status in populations with appreciable differences in mean body weight. The number of anemic individuals in the present study was too small to fully evaluate alternate computational methods.

The estimation of body iron in iron-replete individuals is based on evidence that the serum ferritin concentration correlates closely with body stores as measured either by quantitative phlebotomy or indirectly by nonheme marrow iron concentration or iron absorption.[4] However, the precise quantitative relationship between body iron reserves and serum ferritin has been examined by phlebotomy in only a limited number of subjects. From these studies a linear relationship using a conversion factor of 8 to 10 mg body iron for each μg/L serum ferritin has often been used. However, based on the widely accepted normal range of 12 to 300 μg/L serum ferritin, this would predict that roughly 2.5% of normal individuals have iron stores in excess of 3 g, a figure that seems rather high. Since there is no clearcut evidence that body iron stores are highly skewed in a normal population, we have used a conservative calculation based on a log transformation for serum ferritin although this may underestimate iron stores at high serum ferritin levels. Additional studies are needed to establish the precise relationship between body iron and serum ferritin, especially in the high normal range. Techniques such as magnetic susceptometry may assist in this effort.[23]

The greatest uncertainty exists in estimating body iron in individuals with mild iron deficiency. A scoring system (formula C) was used to relate the degree of abnormality in transferrin saturation, erythrocyte protoporphyrin, and serum ferritin to the deficit in body iron. More sophisticated approaches such as multiple regression analysis might be developed by monitoring the changes in these iron parameters during the gradual induction or alleviation of mild iron deficiency. Further refinements in this calculation might also be developed by studying populations with a higher prevalence of mild iron deficiency. It should be noted that by using a combination of iron parameters, precise cutoff levels for transferrin saturation, erythrocyte protoporphyrin, and serum ferritin are less critical than when using any one of these alone. We observed that 10% to 20% changes in cutoff levels for calculating the index (in formula B) had little effect on calculated iron stores.

One important problem in assessing the iron status of a population is in distinguishing chronic inflammation from iron deficiency. Chronic inflammation assumes greater importance in the elderly and in poorer socioeconomic segments of a population. Clinically, inflammation can be recognized by a low transferrin saturation or elevated erythrocyte protoporphyrin in combination with a high serum ferritin level. It is of interest that the serum ferritin was disproportionately elevated relative to transferrin saturation or erythrocyte protoporphyrin in men and postmenopausal women, suggesting an influence of chronic inflammation in this age group. This effect might be eliminated by excluding individuals from the analysis who have an abnormal transferrin saturation and erythrocyte protoporphyrin but a serum ferritin greater than 50 μg/L.[19] There were too few individuals in the present study (7 out of 2,829) to detect the effect on the frequency distributions.

The ability to estimate iron stores on an individual basis offers certain advantages in survey work. Existing methods are based on the proportion of individuals with one or more abnormal laboratory measurements, an approach that requires large surveys, particularly if the prevalence of iron deficiency is low. Another advantage in estimating iron stores individually is to reduce the time required to plan, conduct, and analyze population surveys, which in the case of NHANES II was nearly a decade. A single technologist can perform the battery of measurements required to estimate iron stores in 20 to 30 individuals daily. This would permit population studies of iron status to be analyzed on an ongoing basis and reduce both the cost and time to conduct them. In addition, individual estimates of body iron provide a measure of variability in iron status and a method of identifying factors that affect iron nutrition in smaller subsets of a population such as method of contraception, intake of iron supplements, socioeconomic status, and bioavailability of dietary iron.

One of the most important advantages in estimating body iron stores is that it defines iron status in the entire population. This has assumed added importance in light of the apparent reduction in the prevalence of iron deficiency between the original HANES conducted in 1973 and NHANES II completed in 1980.[5] Increased consumption of heavily fortified foods and iron supplements have undoubtedly contributed to this improvement. Careful monitoring of the iron status in this country is justified in light of the relatively high frequency of the iron-loading gene which has been reported.[3,24] Although once thought to be a rare disorder, the homozygous state for hereditary hemochromatosis is now believed to occur in approximately 0.5% of this population. This is appreciably higher than the prevalence of iron deficiency anemia observed in adult males in NHANES II. Epidemiologic techniques that are sensitive to changes in the iron-replete as well as iron-deficient segments of the population could assist in drafting regulatory policies relating to iron fortification and supplementation.

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Identification of Platelet Glycoprotein IIb/IIIa as the Major Binding Site for Released Platelet–von Willebrand Factor

By Robert I. Parker and Harvey R. Gralnick

We studied the effects(s) of two monoclonal antibodies, 6D1 and 10E5 (directed against platelet glycoprotein Ib [GPIb] and the GPIIb/IIIa complex, respectively), and purified human plasma fibrinogen on the binding of released platelet-von Willebrand factor (vWf) to the platelet surface. Neither of the monoclonal antibodies nor fibrinogen had any effect on the amount of platelet-vWf expressed on unstimulated platelets or on the amount expressed on platelets stimulated in the absence of extracellular Ca^{++}. However, the antibody directed against GPIIb/IIIa inhibited 72% of the thrombin-induced increase in the platelet-vWf bound to the platelet surface when platelets were stimulated in the presence of 5 mmol/L Ca^{++}. The antibody against GPIb did not inhibit the surface expression of platelet-vWf on stimulated platelets in the presence of Ca^{++}. Purified normal human fibrinogen inhibited the surface binding of platelet-vWf to thrombin-stimulated platelets to a degree similar to that observed with the monoclonal antibody directed against the GPIIb/IIIa complex. These data indicate that platelet-vWf released from platelets binds primarily to the GPIIb/IIIa complex at or near the plasma fibrinogen binding site.

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PLASMA VON WILLEBRAND FACTOR (vWf) binds to the platelet glycoprotein Ib (GPIb) in the presence of ristocetin[1,2] and to the glycoprotein IIb/IIIa (GPIIb/IIIa) complex when platelets are stimulated by adenosine diphosphate (ADP) or thrombin.[3-5] Because fibrinogen competes with vWf for binding to the GPII/IIIa complex, thrombin-induced plasma vWf binding to GPIIb/IIIa is minimal in the presence of purified fibrinogen or in plasma.[5-8] Platelets contain a pool of vWf that is released and bound to the platelet surface after thrombin stimulation.[9-11] The receptor(s) and the sites on the receptors involved in this interaction are unknown. To determine whether either GPIb or GPIIb/IIIa serves as a receptor for released platelet-vWf, we measured the effect of fibrinogen or monoclonal antibodies directed against the platelet glycoproteins Ib and IIb/IIIa on the binding of platelet-vWf to resting and thrombin-stimulated normal human platelets.

MATERIALS AND METHODS

Normal human platelets were prepared from sodium citrate-anticoagulated whole blood using an arabinogalactan (Stractan) gradient as previously described.[12] The platelets were diluted to 200,000/μL in a Tyrode's buffer containing calcium (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.5 mmol/L NaH$_2$PO$_4$ · H$_2$O, 0.9 mmol/L MgCl$_2$ · ^{6}H$_2$O, 5.6 mmol/L dextrose, and 5 mmol/L CaCl$_2$) or the same Tyrode's buffer containing no added CaCl$_2$ and 10 mmol/L EDTA.

An affinity-purified rabbit antihuman vWf antibody was prepared by the method of Hoyer[13] and ^{125}I-radiolabeled by the lactoperoxidase method[14] with the modification that no unlabeled KI was added and the reaction was stopped by the addition of excess phosphate buffer. The anti-vWf antibody preparation used migrated as a single band of 150,000 daltons on 5% polyacrylamide gel electrophoresis,[15] and the radioactivity of this preparation was >95% trichloroacetic acid-precipitable.

Commercially available fibrinogen was purified further by diethylaminoethanol (DEAE) column chromatography from Kabi fibrinogen (grade L, Kabi Diagnostics, Stockholm). One milliliter of lyophilized fibrinogen (10 mg/mL) was run over a 15.5 × 0.8-cm column of Sephacel-DEAE beads (Pharmacia, Uppsala, Sweden) equilibrated with Tris-saline buffer (0.11 mol/L NaCl, 0.01 mol/L Tris-HCl, pH 8.1). The fibrinogen obtained after this procedure was shown to be free of vWf in a radioimmunoassay for vWf[13] and by glyoxyl-agarose gel electrophoresis, followed by autoradiography with an ^{125}I-labeled anti-vWf antibody.[16]

Binding studies. Aliquots (250 μL) of Stractan-separated platelets (200,000/μL) were incubated with anti-vWf antibody (3.75 μg/mL final concentration) at 37 °C for 90 minutes in the presence of monoclonal antibody or fibrinogen. Anti-vWf antibody binding to both resting and stimulated platelets was measured. When platelets were stimulated by human α-thrombin (1.0 U/mL final concentration), the thrombin was added to the platelet (or platelet-monoclonal antibody) suspension immediately before the incubation with the anti-vWf antibody, and incubations were continued for 90 minutes at 37 °C. When a monoclonal antibody was used, the platelets were preincubated with the monoclonal antibody for 15 minutes before incubation with the anti-vWf antibody or thrombin plus anti-vWf antibody.

When the effect of fibrinogen on the platelet binding of anti-vWf antibody was studied, fibrinogen (0.001 to 1.0 mg/mL final concentration) was added to the platelets immediately before the addition of thrombin and anti-vWf antibody (stimulated platelets) or of anti-vWf antibody alone (unstimulated platelets). Hirudin (20 U/mL final concentration) was added to the platelet-fibrinogen-antibody suspension 15 seconds after the addition of α-thrombin to prevent fibrin clot formation.

Incubations were carried out in 1.5-mL polypropylene vials. Free ligand (anti-vWf antibody) was separated from bound ligand by centrifugation at 22 °C (9,000 g for four minutes). The supernatant was removed and the platelet pellet was assayed for bound anti-vWf antibody with an automated gamma counter (Model 1185, Tracor Analytic, Elk Grove, Ill). After correcting for trapping and nonspecific binding of the ^{125}I-labeled antibody to both platelets and the polypropylene vial, the amount of anti-vWf antibody recovered with the platelet pellet was used as a measure of platelet-vWf expressed on the platelet surface (surface-bound platelet-vWf). Because the number of molecules of antibody bound per vWf molecule is unknown, absolute numbers of platelet-vWf molecules expressed on the platelet surface cannot be calculated. However, if the ratio of bound antibody to bound vWf is constant, inferences regarding relative amounts of surface-bound platelet-vWf are valid.

In some experiments, the anti-vWf was omitted and the supernatant (unbound) vWf was analyzed for multimeric structure by

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glyoxal-agarose gel electrophoresis[16] and quantitated by radioimmunoassay.[13] Gels were analyzed by scanning densitometry using an LKB Laser Scanning Densitometer (LKB Model 2202, Bromma, Sweden). In additional experiments, the ability of either monoclonal antibody or purified fibrinogen to displace surface-bound platelet-vWf was measured by first incubating thrombin-stimulated platelets with radiolabeled anti-vWf antibody for 45 minutes at 37 °C before the addition of either monoclonal antibody or fibrinogen. The incubations were continued for an additional 45 minutes (at 37 °C) and then terminated by centrifugation. Molecules of anti-vWf antibody bound per platelet was calculated as described earlier.

Two monoclonal antibodies were used in an attempt to block the binding of platelet-vWf to the platelet surface. Monoclonal antibody 6D1 (designated Stony Brook 1 by the International Committee on Thrombosis and Hemostasis [ICTH]) is directed against a site on platelet GPIb that blocks ristocetin-induced binding of vWf to the platelet and ristocetin-induced platelet agglutination. This antibody has no effect on thrombin-induced platelet aggregation or shape change,[2] and it does not interfere with thrombin binding to platelets.[17] Monoclonal antibody 10E5 (designated Stony Brook 2 by the ICTH) is directed against a site on the GPIIb/IIIa complex that inhibits the ADP-induced binding of fibrinogen to platelets and ADP-, epinephrine-, and thrombin-induced platelet aggregation. This antibody, however, does not inhibit thrombin-induced platelet shape change, only partially inhibits thrombin-induced platelet adenosine triphosphate secretion,[18] and does not interfere with thrombin binding to platelets.[17] Monoclonal antibodies of the same IgG subclasses directed against non-platelet antigens were used as controls in the binding inhibition studies. For anti-GPIb (IgG_1), Coulter Clone (CC) monoclonal antibody T4 (Lot No. 305, Coulter Diagnostics, Hialeah, Fla) was used, and for anti-GPIIb/IIIa (IgG_{2a}), CCI_2 (Lot No. 5AAA3) was used. Coulter clone T4 is a T cell-specific antibody, and CCI_2 is an antibody that recognizes the DR locus on cells.

Buffers were made with deionized distilled water using reagent-grade chemicals. Stractan was purchased from the St Regis Paper Co (Tacoma, Wash); ^{125}I, from New England Nuclear (Boston); hirudin, from Sigma Chemical Co (St Louis); purified human α-thrombin was the kind gift of Dr J. Fenton, New York State Department of Health, Albany; and monoclonal antibodies 6D1 and 10E5 were kindly provided by Dr Barry Coller, State University of New York, Stony Brook.

RESULTS

The presence of thrombin or EDTA in the assay did not alter the antigenicity of plasma vWf as measured by the rabbit antihuman vWf antibody in the liquid-phase radioimmunoassay.[11] Specificity of the anti-vWf antibody was demonstrated by the absence of binding of this antibody to platelets from patients with severe von Willebrand's disease (vWd).[11] Binding of this antibody to normal Stractan-isolated resting platelets was shown to be saturable, reversible, and specific, with steady-state binding achieved by 90 minutes.[11]

Platelet-vWf binding. Neither of the test monoclonal antibodies nor fibrinogen inhibited platelet-vWf expression on unstimulated platelets or on platelets that were thrombin-stimulated in the absence of extracellular Ca^{++} (Table 1). In contrast, the anti-GPIIb/IIIa antibody resulted in a signifi-

Table 1. Effect of Extracellular Calcium, Fibrinogen, or Monoclonal Antibody on the Total and Net Thrombin-Induced Anti-vWf Antibody Binding to Platelets

		10 Molecules Anti-vWf Bound per Platelet					
		5 mmol/L Ca^{++}			No Ca^{++} + EDTA		
Inhibitor	Final Concentration	Unstimulated	Stimulated	Net Thrombin Increase (%)	Unstimulated	Stimulated	Net Thrombin Increase (%)
None	—	4.08	17.42	100	3.55	7.57	30
		(1.39)	(1.03)		(0.61)	(0.24)	
Anti-GPIb	10 μg/mL	4.59	17.04	93	3.80	8.85	38
		(1.30)	(1.40)		(0.89)	(0.67)	
CCT4 (IgG_1)	10 μg/mL	4.61	16.74	91	2.95	7.74	36
		(0.66)	(0.95)		(0.79)	(0.19)	
Anti-GPIIb/IIIa	10 μg/mL	4.05	7.84	28	3.65	7.33	28
		(0.86)	(0.66)		(0.19)	(0.46)	
CCI_2 (IgG_{2a})	10 μg/mL	3.94	15.06	87	3.15	7.93	36
		(0.62)	(2.73)		(0.59)	(1.15)	
Fibrinogen	80 μg/mL	3.93	7.93	30	4.04	8.42	33
		(0.86)	(1.76)		(1.51)	(2.15)	

Values represent the mean (±SD) of six to eight experiments. The net increase in anti-vWf antibody bound to thrombin-stimulated platelets in the presence of 5 mmol/L Ca^{++} (Tyrode's buffer) is defined as 100%. Percentage of net increase in anti-vWf antibody binding is calculated by the formula:

$$\frac{\text{Molecules anti-vWf antibody bound per thrombin-stimulated platelet} - \text{Molecules anti-vWf antibody bound per non-stimulated platelet}}{\text{Molecules anti-vWf antibody bound per thrombin-stimulated Tyrode's (Ca}^{++}\text{) control platelets} - \text{Molecules anti-vWf bound per non-stimulated Tyrode's (Ca}^{++}\text{) control platelets}} \times 100.$$

Example: Table 1, lines 1 and 4:

$$\frac{\text{Anti-GPIIb/IIIa}}{\text{Buffer Control}} \quad \frac{7.84 - 4.05}{17.42 - 4.08} \times 100 = 28$$

cant decrease (55%) in the total platelet-vWf expressed on thrombin-stimulated platelets in the presence of Ca^{++} (Table 1); inhibition was maximal at an antibody concentration of 10 μg/mL. The difference between unstimulated platelet-vWf expression and platelet-vWf expression on thrombin-stimulated platelets is referred to as net thrombin-induced platelet-vWf binding. The anti-GPIIb/IIIa antibody 10E5 inhibited 72% of the net thrombin-induced platelet-vWf binding to platelets in the presence of 5 mmol/L extracellular Ca^{++} (Table 1). Thrombin stimulation of control platelets in the absence of added extracellular Ca^{++} and the presence of EDTA resulted in an increase in platelet-vWf expression of 30% to 40% compared with the increase observed with thrombin-stimulated control platelets in the presence of Ca^{++} (Table 1; last column). This amount of thrombin-induced increase in platelet-vWf binding is similar to the 28% increase seen in thrombin-stimulated platelets in the presence of 10E5 and Ca^{++} (Table 1). Neither anti-GPIb nor control monoclonal antibodies (CCl_2, CCT4) significantly reduced the total expression or net thrombin-induced binding (Table 1) of platelet-vWf to thrombin-stimulated platelets in the presence of extracellular Ca^{++}.

When normal human fibrinogen was incubated with platelets in the presence of extracellular Ca^{++}, marked inhibition of the thrombin-induced increase in platelet-bound vWf was observed (Table 1). The inhibitory effect of fibrinogen was maximal at a final concentration of 80 μg/mL and similar in magnitude to that produced by monoclonal antibody against GPIIb/IIIa (10E5).

The ability of fibrinogen or anti-GPIIb/IIIa to displace surface-bound platelet-vWf was tested by adding purified fibrinogen or 10E5 to suspensions of thrombin-stimulated platelets preincubated with anti-vWf antibody in the presence of 5 mmol/L Ca^{++}. Under these conditions, only small amounts of platelet-vWf was displaced from the platelet surface (Table 2).

Table 2. Displacement of Bound Anti-vWf Antibody From Thrombin-Stimulated Platelets by Monoclonal Antibodies or Fibrinogen

Inhibitor	Concentration (μg/mL)	10^3 Molecules of Anti-vWf Bound/Plt	Displacement (%)
None	—	17.21 (0.25)	0
Anti-GPIb (6D1)	10	16.04 (0.38)	7
IgG_1 control (CCT4)	10	16.28 (0.55)	5
Anti-GPIIb/IIIa (10E5)	10	15.39 (0.54)	11
IgG_{2a} control (CCl_2)	10	16.40 (0.39)	5
Fibrinogen	80	15.49 (0.30)	10

Values represent the mean ± SD of six experiments. Platelets were incubated with radiolabeled anti-vWf antibody in the presence of 5 mmol/L Ca^{++} and thrombin for 45 minutes at 37 °C before the addition of monoclonal antibody or fibrinogen. Incubations then continued for an additional 45 minutes at 37 °C.

Analysis of unbound platelet-vWf. The decrease in thrombin-induced platelet-vWf binding noted in the presence of Ca^{++} and anti-GPIIb/IIIa antibody is accompanied by a commensurate increase in supernatant platelet-vWf (Table 3). The amount of unbound platelet-vWf measured in the supernatants of thrombin-stimulated platelets preincubated with anti-GPIIb/IIIa antibody and Ca^{++} is 45% greater than the amount measured in supernatants of control platelets. Furthermore, platelets stimulated with thrombin in the absence of added extracellular Ca^{++} have a significantly greater amount of supernatant platelet-vWf than do platelets stimulated in the presence of Ca^{++} ($P < .001$, *t* test for comparison of means; Table 2). There is also a slight increase (16%) in supernatant platelet-vWf from platelets thrombin-stimulated in the EDTA-containing buffer in the presence of anti-GPIIb/IIIa antibody. This increase in supernatant platelet-vWf is not reflected by a decrease in bound platelet-vWf antibody.

When the supernatant vWf released from thrombin-stimulated platelets is analyzed by glyoxal-agarose gel electrophoresis (Fig 1), the largest platelet-vWf multimers appear only in the supernatants from platelets suspended in CFT/EDTA (Fig 1, lanes 2, 4, 6, 8). In the presence of monoclonal antibody against GPIIb/IIIa, increased amounts of large vWf multimers, but not the largest platelet-vWf multimers, are present in the supernatants from platelets thrombin-stimulated in the presence of Ca^{++} (Fig 1, lane 3). These findings were confirmed on densitometric analysis of the gels (Table 4). High-molecular-weight vWf multimers (larger than major band 7 on the gels) are significantly increased in the supernatants from platelets stimulated in the presence of Ca^{++} and monoclonal antibody against the GPIIb/IIIa complex (Tyrode's + 10E5) (1.74×10^7 mV sec *v* 1.09×10^7 mV sec, $P < .001$; Table 4).

Table 3. Effect of Extracellular Calcium and Monoclonal Antibodies Against Platelet Glycoproteins on Thrombin-Released Supernatant Platelet-vWf

Monoclonal Antibody (10 μg/mL)	Buffer	R:Ag (vWf) (U/10^{10} Platelets)	Change Over Buffer Control (%)
None	Tyrode's	0.405 ± 0.080	—
	CFT/EDTA	0.802 ± 0.072	—
Anti-GPIb (6D1)	Tyrode's	0.385 ± 0.074	−5
	CFT/EDTA	0.850 ± 0.087	+6
IgG_1 control (CCT4)	Tyrode's	0.416 ± 0.017	+3
	CFT/EDTA	0.865 ± 0.051	+8
Anti-GPIIb/IIIa (10E5)	Tyrode's	0.589 ± 0.069	+45
	CFT/EDTA	0.935 ± 0.064	+16
IgG_{2a} control (CCl_2)	Tyrode's	0.421 ± 0.053	+4
	CFT/EDTA	0.821 ± 0.100	+2

Von Willebrand factor (R:Ag) was measured by radioimmunoassay[13] in supernatant of thrombin-stimulated (1.0 U/mL) arabinogalactan-isolated platelets. Results represent the mean ± S.D. of five to six determinations. Tyrode's buffer contained 5 mmol/L Ca^{++}. CFT/EDTA buffer is a calcium-free Tyrode's buffer containing 10 mmol/L EDTA. The percentage of change represents the increase or decrease in supernatant vWf from an incubation of platelets with monoclonal antibody expressed as a percentage of supernatant vWf from the appropriate platelet/buffer control incubation.

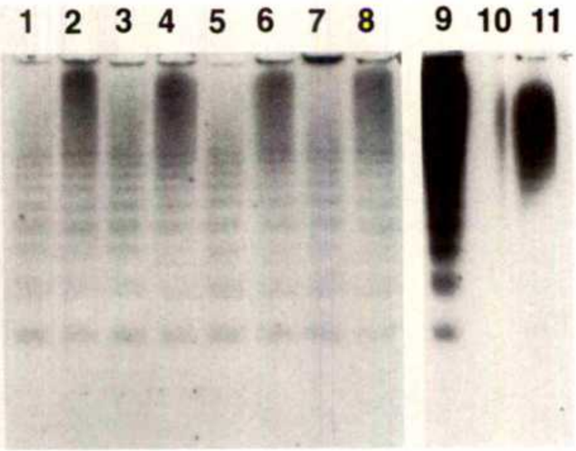

Fig 1. Glyoxyl-agarose gel electrophoresis of unbound supernatant platelet-vWf from thrombin-stimulated platelets (final thrombin concentration 1.0 U/mL) in the presence of monoclonal antibodies in either Ca^{++}-sufficient (Tyrode's) or Ca^{++}-deficient (CFT/EDTA) buffer. Lane 1, Tyrode's + 6D1 (anti-GPIb); lane 2, CFT/EDTA + 6D1 (anti-GPIb); lane 3, Tyrode's + 10E5 (anti-GPIIb/IIIa); lane 4, CFT/EDTA + 10E5 (anti-GPIIb/IIIa); lane 5, Tyrode's + buffer; lane 6, CFT/EDTA + buffer; lane 7, Tyrode's + control monoclonal antibody; lane 8, CFT/EDTA + control monoclonal antibody; lane 9, SDS solubilized platelets; lane 10, vWf-deficient plasma; lane 11, normal plasma.

DISCUSSION

Our data demonstrate that a large percentage of platelet-vWf released from activated platelets binds to the GPIIb/IIIa complex in the presence of extracellular Ca^{++}, while under identical conditions the released platelet-vWf does not bind to (or near) the GPIb site where plasma vWf binds. These findings are consistent with those of Furby et al,[19] who studied the release and subsequent binding of platelet-vWf to normal platelets and platelets deficient in GPIb or GPIIb/IIIa. Furthermore, our data confirm the findings of others[10] showing that the increase in surface platelet-vWf in the presence of Ca^{++} is, in part, the result of preferential binding of the larger platelet-vWf multimers released from thrombin-stimulated platelets. However, we never see the largest platelet-vWf multimers in the supernatants from platelets stimulated with thrombin in the presence of Ca^{++} and monoclonal antibody against GPIIb/IIIa. This inability of anti-GPIIb/IIIa antibody to block the platelet binding of the largest platelet-vWf multimers released suggests that the mobilization and expression of these largest vWf multimers within platelets may occur differently than that of smaller multimers. Furthermore, we see no reduction of platelet-vWf expression on the surface of nonstimulated platelets in the presence of antibodies against either GPIb or GPIIb/IIIa. This finding suggests that the manner of platelet-vWf expression on activated platelets may be different from that observed for nonactivated platelets under steady-state conditions.

Table 4. Densitometric Analysis of Supernatant vWf

		High-Molecular-Weight vWf Multimers	
Lane	Buffer	Area (%)	Density 10^7 mV sec
1	Tyrode's + 6D1	50.17 (4.26)	1.14 (0.10)
2	CFT/EDTA + 6D1	79.49 (5.88)	3.07 (0.23)
3	Tyrode's + 10E5	62.38 (4.46)	1.74 (0.12)
4	CFT/EDTA + 10E5	81.57 (5.43)	3.47 (0.23)
5	Tyrode's	54.08 (5.59)	1.09 (0.11)
6	CFT/EDTA	80.20 (5.89)	2.85 (0.21)
7	Tyrode's + Control McAb	54.68 (5.26)	1.14 (0.11)
8	CFT/EDTA + Control McAb	77.71 (4.96)	2.31 (0.15)

High-molecular-weight multimers are those larger than major band 7 on glyoxal-agarose electrophoresis counting up from the bottom of the gel. This band is indicated by an arrow in Fig 1. The values represent the mean ± SD of four experiments.

Our data also show that in the absence of extracellular Ca^{++}, thrombin increases the platelet-vWf binding to the platelet surface approximately 30% to 40% over the level in nonstimulated platelets. This binding is not inhibited by monoclonal antibodies to GPIb or GPIIb/IIIa or by purified plasma fibrinogen. Thus, approximately 30% to 40% of the platelet-vWf expressed on the platelet surface after (thrombin) activation of platelets is through a mechanism independent of binding to surface-expressed GPIIb/IIIa and independent of extracellular Ca^{++}. The binding of platelet-vWf to thrombin-stimulated platelets in the presence of Ca^{++} and anti-GPIIb/IIIa (10E5) is similar to that seen in thrombin-stimulated control platelets in the presence of EDTA. It is likely that the EDTA effect is mediated through the dissociation of the GPIIb/IIIa complex.[20,21] Measurement of unbound supernatant platelet-vWf from platelets stimulated in buffer without added calcium demonstrates a slight increase when antibody against GPIIb/IIIa is included in the suspension. Under these conditions, we also detect a minor change in the relative multimer distribution of the unbound vWf. It may be that this increase is the consequence of 10E5 inhibition of platelet-vWf binding to the small number of GPIIb/IIIa complexes not dissociated in the 10 mmol/L EDTA buffer.

A possible explanation of these data is that as platelets encounter agonists in circulation, limited release reactions occur, resulting in redistribution of small amounts of α-granule platelet-vWf to the cannicular system. This vWf may be tightly bound to GPIIb/IIIa complexes that are not effectively exposed to the Ca^{++}-free, EDTA-containing buffer on isolation and resuspension of the platelets. These vWf multimers bound to the deep canalicular membrane may not be accessible to the anti-vWf antibody under resting conditions, but on stimulation of the platelets, consequent shape change and membrane perturbations render them "visible" to our antibody probe. This hypothesis would suggest that approximately 30% to 40% of the vWf subsequently expressed on the surface of stimulated platelets resides in extragranular sites. This explanation has the advantage of obviating the need to describe a third binding site for vWf.

The physiologic significance of thrombin-induced plasma-vWf binding to the GPIIb/IIIa complex has been questioned when one considers the great molar excess of fibrinogen over vWf in plasma.[5,7,8] Our findings that released platelet-vWf also binds primarily to GPIIb/IIIa and that platelet-vWf bound to GPIIb/IIIa is not readily displaced by either purified fibrinogen or anti-GPIIb/IIIa monoclonal antibody suggest that this receptor-ligand interaction may be significant under conditions where platelet-vWf enjoys a local concentration excess (eg, platelet activation and release occurring on or near exposed subendothelium). This hypothesis may also explain why added vWf does not augment thrombin-induced platelet aggregation in vitro,[6] as the GPIIb/IIIa binding sites for vWf would already be occupied by released/rebound platelet-vWf. It may be that the thrombin-induced exposure of this vWf binding site enhances the platelets' interaction with other platelets and endothelial/subendothelial surfaces.

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On the Identity of Vitronectin and S-Protein: Immunological Crossreactivity and Functional Studies

By Bianca R. Tomasini and Deane F. Mosher

Vitronectin (serum spreading factor), a major serum cell adhesion molecule, was compared with S-protein, the inhibitor of the C5-9 membrane attack complex. Data from the literature indicate that S-protein and vitronectin are alpha globulins with the same aminoterminal residues, amino acid compositions, and concentrations in normal plasma (150 to 250 μg/mL). Both proteins have been reported to interact with the thrombin–antithrombin complex. The cDNA sequences of vitronectin and S-protein were recently determined and found to be almost identical. In the present studies, rabbit-anti–S-protein and a monoclonal antibody to vitronectin both recognized 65,000- and 75,000-molecular weight (mol wt) polypeptides when plasma or serum proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The 65,000 and 75,000-mol wt polypeptides bound more avidly from serum than plasma to monoclonal anti-vitronectin or heparin coupled to agarose. The presence or absence of the polypeptides constituted a major difference between the heparin-binding proteins of serum and plasma. When complement-activated serum and unactivated serum were separated by gel filtration, vitronectin coeluted with C9 in high-mol-wt fractions of activated serum but not unactivated serum. Purified S-protein was recognized by the monoclonal antibody to vitronectin and promoted spreading of human skin fibroblasts. Both vitronectin and S-protein were degraded by thrombin. On the basis of immunological and functional, as well as biochemical, properties, therefore, S-protein and vitronectin are the same.

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VITRONECTIN (SERUM SPREADING FACTOR) is responsible for most of the cell spreading activity present in serum.[1] Vitronectin is an alpha globulin[2] that has been isolated in the form of two noncovalently associated polypeptides with molecular weights (mol wt) of 75,000 and 65,000.[3,4] Like the other cell attachment protein of serum fibronectin, vitronectin has been localized in the extracellular matrices of various tissues[4,5] and cells in culture[4] and circulates in plasma at a concentration of 250 μg/mL.[6] The cDNA of vitronectin has been sequenced, and the deduced amino acid sequence contains the tripeptide arg-gly-asp, which is known to be a necessary recognition sequence for cellular adhesion to several adhesive proteins.[7] Recent studies by Pytela et al have described a presumptive cellular adhesion receptor for vitronectin that has two components with mol wt of 125,000 and 115,000.[8]

It was found by Suzuki et al[9] and Barnes et al[10] that the sequence of the first 44 amino acids at the NH_2-terminal of vitronectin was identical to the sequence of Somatomedin B, a polypeptide previously believed to have growth-promoting capabilities. Due to the relatively high concentration of vitronectin in plasma and serum, it seemed probable to us that vitronectin itself also had been characterized before. Although we compared the properties of vitronectin with a number of 60,000- to 80,000-mol wt glycoproteins, we found no similarities. At the suggestion of Dr John Griffin (Dept of Immunology, Research Institute of Scripps Clinic, La Jolla, Calif), we tested the possibility that S-protein of the complement system might be similar to vitronectin.

S-protein, as characterized by Podack and Müller-Eberhard,[11] is a glycoprotein that binds to C5b-7. The soluble complex then incorporates C8 and C9. By promoting the formation of this soluble complex rather than the lytic membrane attack complex, S-protein is thought to inhibit (modulate) complement-mediated cell lysis. The second role associated with S-protein is that of binding to the thrombin–antithrombin III complex[11-13] in a manner that slows down the formation of the thrombin–antithrombin III complex, thereby resulting in the protection of thrombin from inactivation by antithrombin III. The thrombin–antithrombin III complex, once formed, binds tightly to S-protein.

When we compared various properties of vitronectin and S-protein reported in the literature, we found many similarities. S-protein, like vitronectin, consists of two polypeptides with mol wt in the range of 65,000 and 80,000,[14] is present in plasma at concentrations of 200 μg/mL, and has the electrophoretic mobility of an alpha globulin.[11] It has the same NH_2-terminal residue as vitronectin.[14] The glass-binding properties of vitronectin have been well documented.[4,15] S-protein has been reported to adsorb extensively onto other surfaces, and thus the yields of published purifications are less than 10%.[14] Ill and Ruoslahti recently found that vitronectin binds to the thrombin–antithrombin III complex in a manner similar to that described for S-protein.[16] Finally, when we compared the reported amino acid compositions of vitronectin and S-protein,[3,14] we found extensive similarities. No residue was different by more than 10%.

In the present paper we show that S-protein and vitronectin are immunologically and functionally the same. While these studies were being prepared for publication, the

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sequence of a cDNA for S-protein was published and shown to be nearly identical to cDNA for vitronectin.[17]

MATERIALS AND METHODS

Column chromatography of plasma and serum. All column chromatography was carried out at room temperature. Platelet-poor fresh frozen plasma was obtained from the Red Cross (Madison, Wis), and serum was obtained after synerisis of clots formed from recalcified citrated plasma (20 mmol/L $CaCl_2$).

Chromatography of plasma or serum on monoclonal-anti-vitronectin (MaVN)-Sepharose column was done as described by Suzuki et al.[9] Hybridoma cells producing MaVN[4] were obtained from Dr E. Hayman (La Jolla [Calif] Cancer Research Foundation), and MaVN IgG was prepared by chromatography of spent media or mouse ascites on protein A-Sepharose (Sigma Chemical Co, St Louis). MaVN IgG was coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. A column of MaVN-Sepharose (2 × 2.5 cm) was equilibrated with phosphate-buffered saline, pH 7.0, before application of plasma or serum (2 mL), and bound material was eluted with 8 mol/L urea, 50 mmol/L Tris, pH 7.0. Protein elution was monitored by absorbance at 280 nm, and peak fractions were collected in polypropylene tubes.

Heparin-agarose (Bio-Rad, Richmond, Calif) columns (1 × 6 cm) were equilibrated with Tris-buffered saline, 1 mmol/L EDTA, pH 7.4. Bound protein from 1 mL plasma or serum was eluted with 600 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4.

As a control, plasma and serum samples were chromatographed on Sepharose 4B columns under conditions described earlier, and effluent volumes corresponding to peak fractions were saved and analyzed.

Immunoblotting. Rabbit antihuman S-protein (RaS-protein) and rabbit antihuman C9 (RaC9) were purchased from Calbiochem (San Diego). Samples were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gels[18] and then electroblotted[19] onto nitrocellulose filters (Scleicher & Schuell, Keene, NH). One section of the blot was stained with 0.1% naphthol blue black in 45% methanol and 10% acetic acid to visualize protein. Replicate sections were soaked in Tris-buffered saline containing 3% bovine serum albumin for one hour at 37 °C, rinsed in Tris-buffered saline, and incubated overnight in Tris-buffered saline containing 1% normal goat serum and 1% appropriate rabbit antiserum or 10% spent medium from MaVN hybridoma cells. After incubation with primary antibody, blots were rinsed in Tris-buffered saline and incubated for one hour at room temperature in Tris-buffered saline containing 1% normal goat serum and 1% peroxidase-conjugated antirabbit or antimouse IgG (Cappel Laboratories, Westchester, Pa). Blots were rinsed again in Tris-buffered saline and washed in substrate solution (Polysciences, Warrington, Pa; Kirkegaard and Perry, Gaithersburg, Md).

Chromatography of zymosan-activated serum. Zymosan (Sigma) was suspended in Tris-buffered saline containing 2 mmol/L Ca^{++} and 2 mmol/L Mg^{++}. Either 0.5 mL of zymosan suspension (100 mg/mL) or buffer alone was added to 5 mL serum, and the mixtures were incubated for four hours at 37 °C in the presence or absence of 20 mmol/L EDTA. Samples were centrifuged at 9,000 g for ten minutes and supernatants (0.5 mL) were separated on Bio-Gel A-15m or A-1.5m columns (1.3 × 18 cm). Columns were equilibrated and run with Tris-buffered saline containing 2 mmol/L Ca^{++} and Mg^{++} at room temperature. Fractions (0.9 mL) were collected in polypropylene tubes and analyzed by electrophoresis and immunoblotting as described.

Cell adhesive activity of purified S-protein. S-protein was purified as described by Dahlbäck and Podack.[14] The purification was monitored by SDS-PAGE, and at each step, the fractions predicted to contain S-protein contained 65,000- and 75,000-mol wt polypeptides that were recognized by MaVN on immunoblots. Contaminants present in the preparation obtained after the Sephacryl S-200 chromatography step were removed by chromatography on concanavalin A-Sepharose 4B[3] (Sigma) and gelatin-agarose (Sigma). Cell attachment and spreading activity of the final preparation was tested by coating 22-mm-diameter wells in microtiter plates (Costar, Cambridge, Mass) with 0.2 mL of a 10-μg/mL protein solution (one hour incubation). The wells were washed and incubated with 0.3% bovine serum albumin, washed again, and incubated with a 2×10^5 cells per milliliter suspension of cultured foreskin fibroblasts (A1-F) in serum-free F-12 media for one hour at 37 °C. Wells coated with fibronectin, vitronectin purified by the method of Hayman et al,[4,9] and albumin were used as positive and negative controls. Adherent cells were stained with Coomassie brilliant blue, and replicate 0.6-mm^2 fields were counted.

Thrombin cleavage of vitronectin and S-protein. Vitronectin and S-protein (purified as described) were incubated with a 1.6 molar excess of alpha thrombin (a generous gift from John Fenton II, New York State Department of Health, Albany) for two hours at 37 °C, and the reaction was stopped by addition of the synthetic thrombin inhibitor PPACK (Calbiochem). Fragmentation was monitored by SDS-PAGE analysis and immunoblotting with MaVN.

RESULTS

As shown in Fig 1, RaS-protein was polyspecific when assayed by immunoblotting of plasma and serum; two of the proteins recognized were serum albumin and IgG, reactions we also found with several other commercial antisera. Two major polypeptides were recognized by the antiserum. The two polypeptides migrated with the same mobilities as the polypeptides recognized by MaVN and reacted with the same proportional intensities.

As shown in Fig 2, when plasma (lane 1) and serum (lane 2) samples were chromatographed on a MaVN-Sepharose column (lanes A) on a heparin-agarose column (lanes B), and, as a control, on a Sepharose column (lanes C), the polypeptides recognized by MaVN bound to MaVN and heparin out of serum but not out of plasma. The antiserum to S-protein recognized two polypeptides with identical migration in SDS gels as those recognized by MaVN and with the same proportional intensities. In other studies, we found that if plasma was recalcified in the presence of thrombin inhibitor and then passed over a heparin column, vitronectin did not bind[20] (not shown).

Mimicking experiments reported for S-protein by Podack et al,[21] we separated zymosan-activated serum or unactivated serum by gel filtration on Biogel A-15m and compared the elution patterns of vitronectin and C9 (Fig 3). The lane numbers in Fig 3B represent every fourth fraction of the elution profile shown in Fig 3A, beginning with the void volume. C9 was identified in high-mol-wt fractions (shown in lanes 2 through 5) of zymosan-activated serum. The equivalent fractions of unactivated serum (lanes 2 through 5) did not contain C9. Monomeric C9 would be expected to elute with albumin; however, the presence of albumin in the fractions shown in lanes 6 through 9 distorts the C9 staining. Immunoblotting of a replicate gel of activated serum with MaVN demonstrated vitronectin in the same high-mol-wt

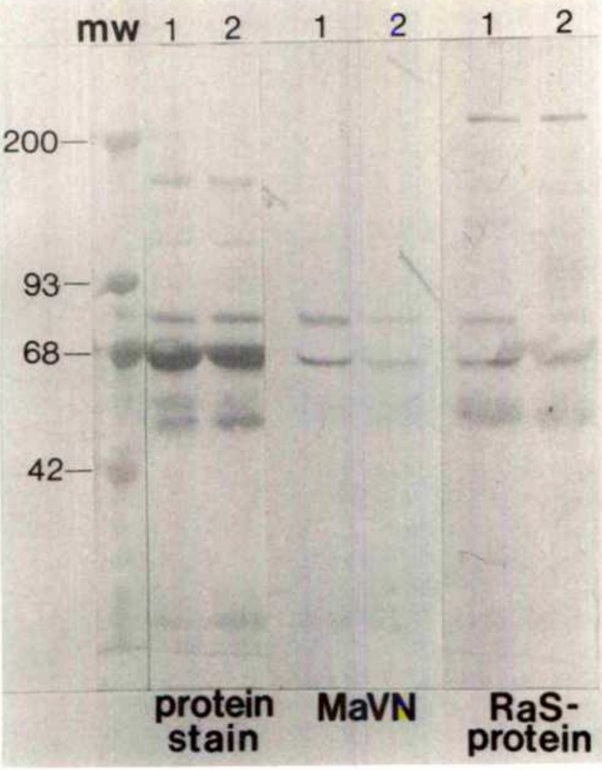

Fig 1. Immunoblots of plasma and serum with MaVN and RaS-protein. Reduced plasma (lane 1) and serum (lane 2) proteins were separated by SDS-PAGE and transferred onto nitrocellulose filters. Part of the blot was stained for protein with 1% naphthol blue black; replica blots were incubated with MaVN (10% spent media) or 1% RaS-protein, followed by the appropriate peroxidase-conjugated secondary antibody to visualize antibodies bound.

fractions in which C9 was detected (lanes 3 through 5). Vitronectin in unactivated serum or in serum treated with EDTA followed by zymosan (not shown) was found only in the fractions containing lower-mol-wt proteins (lanes 5 through 9). That is, vitronectin was present in eight fractions (7.2 mL) of separated activated serum that did not contain vitronectin in unactivated serum (indicated by bar in Fig 3A). The finding that vitronectin co-eluted with C9 in high-mol-wt fractions of zymosan-activated serum was reproducible and also evident when serum samples were chromatographed on Bio-Gel A-1.5m (not shown). RaS-protein, like MaVN, detected the 65,000- and 75,000-mol wt polypeptides in the high-mol-wt fractions of activated but not unactivated serum (not shown).

S-protein purified by the method of Dahlbäck and Podack[14] was tested for spreading factor activity. Trace amounts of fibronectin (approximately 0.2%) and albumin detectable by immunoblotting in the preparation after the Sephacryl S-200 gel filtration step were removed by chromatography on concanavalin A-Sepharose 4B and gelatin agarose. The protein obtained was recognized by MaVN and RaS-protein (not shown) and was active in a cell spreading assay (Table 1).

Podack and Müller-Eberhard[11] reported the lack of susceptibility of S-protein to cleavage with a twofold molar excess of thrombin. In contrast, Silnutzer and Barnes[22] have reported the cleavage of serum spreading factor (vitronectin) to a 57,000-mol wt fragment produced by incubation with thrombin (observed with a 0.15–1.3-fold molar ratio of thrombin to spreading factor) for two hours at 37 °C. As shown in Fig 4, when vitronectin (lane 1) or S-protein (lane 2) was incubated with thrombin (lanes 3 and 4, respectively), a major 60,000-mol wt fragment was obtained (indicated by

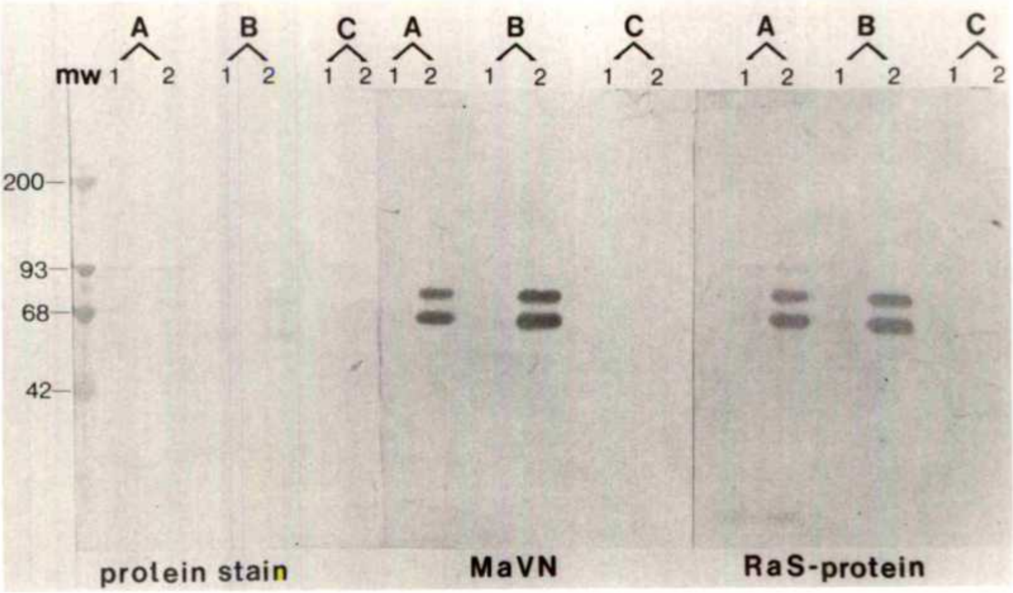

Fig 2. Immunoblots of affinity chromatography-separated plasma and serum against MaVN and RaS-protein. Shown here are the bound fractions of plasma (lanes 1) and serum (lanes 2) chromatographed on MaVN-Sepharose (lanes A), heparin-agarose (lanes B), and Sepharose (lanes C). Samples were analyzed as described in Fig 1.

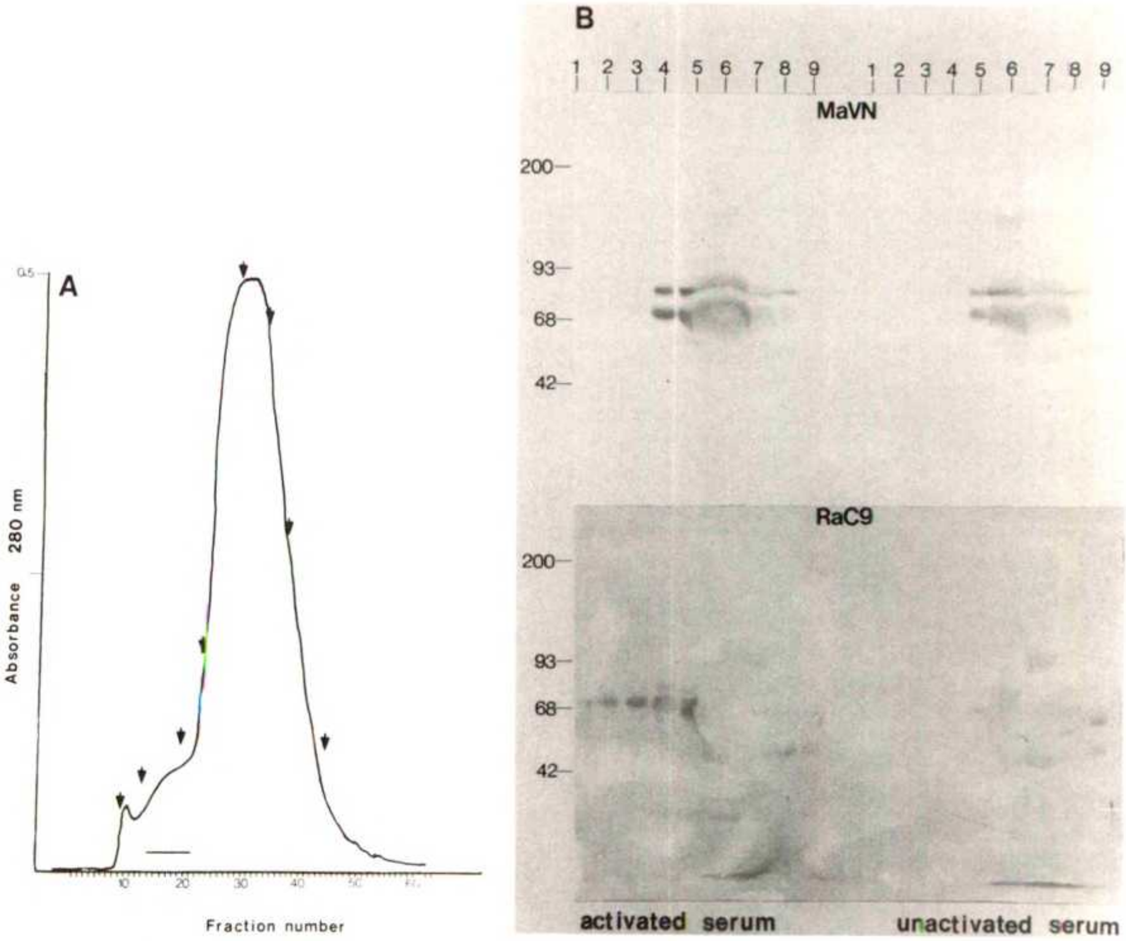

Fig 3. Separation of zymosan-activated serum and unactivated serum by gel filtration on Bio-Gel A-15m. (A) Protein elution profile for 0.5 mL activated serum (the same profile was obtained for unactivated serum). Arrows indicate the positions of fractions used in immunoblotting of both activated and unactivated serum. The line indicates the fractions where VN was detected in activated but not unactivated serum. (B) Immunoblots of fractions from zymosan-activated serum and unactivated serum reacted against MaVN (above) and RaC9 (below), followed by the appropriate peroxidase conjugates.

arrowhead). This fragment was detected by immunoblotting with MaVN and by protein stain (not shown).

DISCUSSION

We found that a commercial rabbit antiserum to S-protein and a monoclonal antibody against vitronectin recognized polypeptides with the same migration on SDS gels. The 65,000- and 75,000-mol wt polypeptides recognized by these antibodies bound avidly to MaVN-Sepharose and heparin-agarose out of serum but not out of plasma. The differential binding to heparin-agarose is specific to vitronectin/S-protein, as compared with two other heparin-binding glycoproteins, fibronectin or histidine-rich glycoprotein,[20] and is probably due to complex formation with thrombin–antithrombin III.[11-13,16] Vitronectin was incorporated into a large complex when serum was activated by zymosan. Finally, we found that MaVN recognized the 65,000- and 75,000-mol wt polypeptides obtained from the purification protocol described by Dahlbäck and Podack for S-protein[14] and that S-protein obtained by this purification was equivalent in spreading factor activity to vitronectin obtained by the purification described by Hayman et al.[4,9]

Table 1. Spreading Factor Activity of S-Protein

	Cells per mm^2 (x ± SEM, n = 4)	
Protein Coating	Attached Only	Attached and Spread
S-protein	20 ± 10	112 ± 12
Vitronectin	28 ± 8	117 ± 12
Fibronectin	47 ± 14	148 ± 20
Albumin	67 ± 13	≤1

Jenne and Stanley[17] recently sequenced the cDNA for S-protein obtained from a human liver cDNA pEX library and found it to be similar, although not identical, to the sequence of the cDNA of vitronectin obtained from a human liver cDNA λgtll library reported by Suzuki et al.[7] There were discrepancies in the nucleotide sequences of the two proteins that produced differences in amino acids 142-6, 206, 347, 381, and 423.[7,17] There were also conservative nucleo-

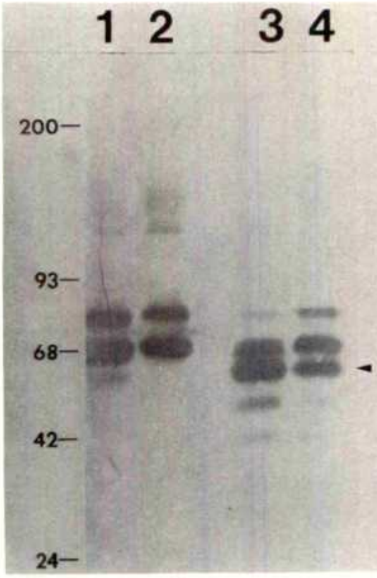

Fig 4. Immunoblot of vitronectin and S-protein digestion by thrombin with MaVN. Vitronectin or S-protein (15 μg) were incubated with thrombin (11 μg) for two hours at 37 °C. Incubation mixtures were subjected to SDS-PAGE, transferred onto nitrocellulose filters, and treated as described in Materials and Methods with 10% MaVN spent media, followed by 1% goat antimouse peroxidase conjugate. Lane 1, purified vitronectin; lane 2, purified S-protein; lane 3, vitronectin incubated with thrombin; lane 4, S-protein incubated with thrombin. Arrowhead indicates a major proteolysis fragment.

tide changes. It has been reported that vitronectin is cleaved by thrombin,[22] whereas S-protein is not.[11] However, we found that both proteins are susceptible to thrombin cleavage. Thus, the data summarized in the introduction and presented in Results are strong evidence that vitronectin is the same as S-protein and that the discrepancies in cDNA sequences probably represent sequencing artifacts.

The biological functions of vitronectin/S-protein must be diverse. Others have discussed possible thrombin and trypsin cleavage sites in this protein and the implications for heparin-binding, promotion of cell attachment, and the release of the Somatomedin B polypeptide.[7,17,22] It will be interesting to determine if regulatory conformational changes also enable vitronectin/S-protein to function specifically in different physiological systems. One example of a conformational change that may take place is that apparently induced by binding to the thrombin–antithrombin III complex.[11–13,16] This binding may expose an otherwise buried heparin-binding site in vitronectin.[20,23] Vitronectin/S-protein in the ternary complex could then sequester heparin and thus block the ability of heparin to further accelerate thrombin–antithrombin III complex formation, as reported by Preissner et al.[13]

ACKNOWLEDGMENT

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Characterization of a Platelet Membrane Protein of Low Molecular Weight Associated With Platelet Activation Following Binding by Monoclonal Antibody AG-1

By Jonathan L. Miller, John M. Kupinski, and Kent O. Hustad

With the exception of the major platelet glycoproteins IIb/IIIa and Ib, which function as receptors for fibrinogen and von Willebrand factor, little is presently known regarding the possible role of other platelet surface proteins in mediating platelet aggregation. We report the production of a murine monoclonal antibody (AG-1) recognizing human platelet membrane surface protein of relatively low molecular weight (mol wt) that may be involved in this process. AG-1 added to human platelet-rich plasma induces dense granule secretion and aggregation, with lag phase and maximal extent of aggregation dependent on antibody concentration. Aggregation induced by AG-1 is inhibited by AG-1 Fab fragments, indicating that the response is not Fc receptor-mediated. Although AG-1 continues to produce platelet shape change in the presence of EDTA, aggregation is fully inhibited and appears to be mediated by fibrinogen binding to glycoproteins IIb/IIIa. AG-1 is a potent stimulus of thromboxane formation, but full inhibition of thromboxane production by 30 μmol/L indomethacin does not significantly inhibit platelet aggregation induced by 25 μg/mL AG-1, indicating that aggregation induced by AG-1 may proceed by way of an endoperoxide-independent pathway. Quantitation of AG-1 Fab binding to platelets reveals approximately 65,000 binding sites per platelet. When intact platelets are radioiodinated, immunoprecipitation of NP-40 lysates by AG-1 reveals an intensely labeled protein with an apparent mol wt of ~21,000 daltons, and several additional bands in the mol wt range of 22,000 to 28,000 daltons, all sharing the AG-1 epitope. These bands appear to be distinct from glycoprotein IX or from the β-chains of glycoprotein Ib or IIb. Finally, studies with platelets labeled by the periodate-[^{3}H]borohydride procedure suggest the possibility of complex formation between subpopulations of glycoprotein Ib and the low-mol-wt glycoproteins recognized by AG-1.

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THE PLATELET MEMBRANE is the focus for molecular interactions involved in platelet activation and the platelet adhesion and aggregation responses. Platelet membrane glycoprotein Ib (GPIb) serves as the receptor for von Willebrand factor and is responsible for platelet adhesion to vascular subendothelium.[1,2] The membrane complex GPIIb/IIIa serves as the fibrinogen receptor and is thought to mediate platelet aggregation.[1,2] In contrast to these membrane glycoproteins, relatively little is presently known concerning the receptors for the various agonists that regulate platelet function and the signal-transducing elements of the membrane.

Studies using radiolabeled ligands have helped to establish that platelet membranes have specific receptors for a number of platelet stimuli, including thrombin, adenosine diphosphate (ADP), serotonin, epinephrine, and collagen.[3] However, the membrane proteins that serve as the specific, functional receptors for these agents have not been conclusively identified. Bennett et al[4] have identified a 100,000-dalton membrane-associated protein that is covalently labeled by the ADP affinity analog 5′-p-fluorosulfonylbenzoyl adenosine (FSBA). Incubation of platelets with FSBA resulted in the loss of platelet responsiveness to ADP, suggesting that the 100,000-dalton polypeptide was the ADP receptor involved in platelet activation. Studies with α-thrombin have suggested that the platelet membrane may have two distinct receptors. GPV has been shown to be a thrombin substrate that is preferentially cleaved from the platelet membrane.[2,5,6] However, McGowan et al[7] have found no consistent correlation between the degree of GPV hydrolysis and the extent of platelet activation. GPIb binds thrombin, and its absence in patients with Bernard-Soulier syndrome and modulation of its expression either by proteolysis or with antibodies correlate with decreased platelet responsiveness to α-thrombin.[2,8] However, for both ADP and α-thrombin, a direct causal relationship between ligand binding to a specific membrane protein and platelet activation has not been demonstrated.

The present study focuses on membrane glycoproteins of relatively low molecular weight (mol wt) that may play a role in the processes leading to platelet activation. A new monoclonal antibody, AG-1, that recognizes such surface protein(s) and is itself able to induce activation of platelets is described, and the membrane protein(s) characterized.

MATERIALS AND METHODS

Platelet function studies. For studies of platelet aggregation and adenosine triphosphate (ATP) secretion on citrated platelet-rich plasma (PRP), blood was drawn from normal volunteers and lumi-aggregation studies performed as previously described.[9] Studies on washed platelets were performed on platelets prepared by the albumin density gradient method of Walsh et al.[10] For studies of 5-hydroxytryptamine secretion, PRP prepared from blood collected in acid-citrate-dextrose was incubated for 30 minutes at 37 °C with 2 μmol/L [^{14}C]5-hydroxytryptamine creatinine sulfate (Amersham, Arlington Heights, Ill) before washing and final resuspension in calcium- and magnesium-free Hanks solution (CMF-Hanks), pH 7.3, to which was added 1 mmol/L calcium (except in experiments where EDTA was used). Radioimmunoassay for thromboxane B_2 generation by platelets in citrated PRP under nonstirred conditions

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was performed by the method of Granstrom and Kendahl[11] as modified by Walenga et al.[12]

Production of AG-1 monoclonal antibody and Fab fragments. BALB/c mice were immunized with washed platelets from patients previously diagnosed as having platelet-type von Willebrand's disease.[13] Spleen cell suspensions prepared from the immunized mice were fused with P3 NS1/Ag 4.1 myeloma cells. Two weeks after fusion, culture supernatants were screened for their capacity to inhibit asialo von Willebrand factor-induced platelet agglutination of patient platelets in the presence of 5 mmol/L EDTA.[14] Several culture supernatants were able to augment this response and on further testing were found capable of inducing aggregation of normal platelets without the addition of other agonists. One of these hybridomas (AG-1) was cloned by limiting dilution. The antibody was produced in ascites fluid of pristane-primed BALB/c mice and partially purified by precipitation with 50% saturated ammonium sulfate. After dialysis in phosphate-buffered saline (PBS), it was further purified by affinity chromatography on Affi-Gel Protein A (MAPS, Bio-Rad, Richmond, Calif). The immunoglobulin class of AG-1 was determined by Ouchterlony analysis using antisera purchased from Serotec (Bicester Oxon, England) and found to be an IgG of the $\gamma 1$ subclass. Murine monoclonal antibodies AP-1 and AP-2, recognizing human platelet glycoproteins Ib and IIb/IIIa respectively,[15] were a kind gift of Dr Thomas Kunicki of the Southeastern Wisconsin Blood Center, Milwaukee.

For the preparation of Fab fragments, AG-1 monoclonal antibody at a concentration of 2.5 mg/mL was first digested with 25 μg/mL pepsin. After one hour at 37 °C, the digestion was stopped by adding $\frac{1}{10}$ vol of 3 mol/L Tris, pH 9.0. The digest was reduced with 10 mmol/L cysteine and then alkylated with 25 mmol/L iodoacetamide before fractionation on a Sephadex G-150 superfine column (1.5 × 80 cm). The final AG-1 Fab pool was free of undigested IgG by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Indirect immunofluorescence. Peripheral blood mononuclear cells isolated by Ficoll-Hypaque density centrifugation or washed platelets at concentrations of 10^7/mL were incubated with the primary monoclonal antibody or normal mouse serum at immunoglobulin concentrations of approximately 10 μg/mL for 30 minutes at 0 °C. After incubation with fluorescein-conjugated $F(ab')_2$ fragments of goat antimouse IgG (Cappel, Malvern, Pa) and multiple washes, the cells were analyzed on a fluorescence-activated cell sorter (Coulter Epics V, Coulter Electronics, Hialeah, Fla).

Scanning electron microscopy. Platelets in citrated PRP were prepared for scanning electron microscopy by fixation in 2.5% glutaraldehyde in White's buffered saline[16] for 20 minutes at room temperature. The fixed platelets were then centrifuged onto glass cover slips,[17] rinsed in 0.9% NaCl, and dehydrated in graded ethanol solutions. Samples were transferred to acetone for five minutes and then critical point dried in carbon dioxide. The dehydrated samples were coated with gold and palladium using a Technics Hummer II (Technics, Alexandria, Va). Specimens were examined with a Hitachi scanning electron microscope at 25 kV.

AG-1 Fab binding. AG-1 Fab fragments were iodinated by the method of Fraker and Speck[18] using Iodogen (Pierce Chemical Co, Rockford, Ill). ^{125}I-Iodinated Fab was separated from free ^{125}I on a 10-mL Sephadex G-25 column equilibrated with PBS. The specific activity of the Fab was 1,550 cpm/ng protein as determined by absorbance at 280 nm.

After incubation of ^{125}I-AG-1 Fab with platelets in PRP for 30 minutes at room temperature, bound ^{125}I-AG-1 Fab was separated from free by centrifugation of the platelets through 20% sucrose in CMF-Hanks containing 2% bovine serum albumin. Nonspecific binding was assessed by measuring binding in the presence of 800 μg/mL undigested AG-1 antibody. The number of ^{125}I-AG-1 binding sites per platelet was obtained by Scatchard analysis of the binding data.[19]

Radiolabeling of platelets and immunoprecipitation of platelet lysates. To citrated PRP obtained from normal volunteers was added EDTA (13 mmol/L, final concentration), and the platelets were then washed three times in medium containing 154 mmol/L NaCl and 1 mmol/L EDTA, buffered to pH 7.4 with 10 mmol/L Tris. Platelets (10^9) were iodinated by the lactoperoxidase procedure with 1 mCi of Na^{125}I, as described by Phillips and Agin[20] or labeled by the periodate-[^{3}H]borohydride procedure.[21]

For immunoprecipitation studies, labeled platelets at a concentration of 0.5 to 1.0 × 10^9/mL were solubilized with 0.5% (vol/vol) Nonidet P40 (NP-40) in the presence of iodoacetamide (10 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), aprotinin (1%), and soybean trypsin inhibitor (25 μg/mL) (all obtained from Sigma Chemical Co, St Louis) to minimize proteolysis. The detergent lysates were centrifuged at 100,000 g for 60 minutes before immunoprecipitation. Lysates were incubated with 30 μg of the monoclonal antibody for 18 hours at 4 °C. The antibody and immune complexes were precipitated with goat antimouse immunoglobulin conjugated to agarose beads (Sigma). The beads were washed exhaustively in Tris-buffered saline with 0.1% NP-40 and the bound material eluted in 5% SDS buffered to pH 6.8 with 10 mmol/L Tris.

Electrophoretic analysis. One- and two-dimensional nonreduced-reduced SDS-PAGE of radiolabeled whole platelet lysates and immunoprecipitates was carried out using the discontinuous buffer method of Laemmli.[22] ^{125}I-Labeled polypeptides were visualized by autoradiography with Kodak XRP-1 film and a Dupont Cronex intensifying screen (Dupont, Wilmington, Del). ^{3}H-Labeled polypeptides were detected by fluorography after impregnating the gel with Enhance (New England Nuclear, Boston). Molecular weights were established by running marker proteins in parallel, with subsequent detection by Coomassie brilliant blue staining.

Electroimmunoblotting of platelet membrane proteins separated by SDS-PAGE was performed as described by Towbin et al.[23] Proteins were transferred in 25 mmol/L Tris, 192 mmol/L glycine, 20% (vol/vol) methanol, pH 8.3 onto a nitrocellulose sheet. After blocking of the nitrocellulose sheet in 50 mmol/L Tris, 150 mmol/L NaCl, 0.05% (vol/vol) Tween 20, pH 7.3 (Bio-Rad, Richmond, Calif), the sheet was incubated first in AG-1 and then in peroxidase-conjugated goat antimouse IgG (Hyclone Labs, Logan, Utah). Peroxidase activity was visualized with 0.015% H_2O_2 (wt/vol) and 0.5 mg/mL 4-chloro-1-napthol (Sigma) in Tris-buffered saline.

RESULTS

Cellular specificity. Monoclonal antibody AG-1 was prepared by immunizing BALB/c mice with washed platelets from patients having platelet-type von Willebrand's disease, a disorder of hemostasis in which the platelet membrane shows an abnormally enhanced binding of von Willebrand factor.[13,24] Although originally prepared against patient platelets, AG-1 has been found to be reactive with the platelets of all persons tested to date (>75).

Binding of AG-1 to washed platelets from human subjects was demonstrated by indirect immunofluorescence. The platelets from normal subjects displayed a log normal distribution of fluorescence. The degree of staining was comparable to that produced by the monoclonal antibody AP-2, which recognizes the glycoprotein complex IIb/IIIa. Examination of peripheral blood lymphocytes and monocytes from the same individuals failed to reveal significant labeling.

Bone marrow aspirates were also examined microscopically by the indirect immunoperoxidase technique; only megakaryocytes and platelets stained with AG-1.

Characterization of AG-1–induced aggregation, secretion, and thromboxane B_2 production. The addition of AG-1 antibody to PRP resulted in strong platelet aggregation, ATP secretion, and thromboxane formation. At lower concentrations of AG-1, both aggregation and dense granule secretion were preceded by a characteristic lag phase (Fig 1). Over the concentration range of approximately 1.6 to 25 μg/mL (or higher), the total extent of platelet aggregation varied only slightly, although the lag phase varied considerably. Aggregation induced by 20 μg/mL AG-1 was not accompanied by lactate dehydrogenase release, indicating a noncytolytic mechanism for the aggregation and secretion responses. AG-1–induced platelet aggregation in PRP was not blocked by 30 μmol/L indomethacin, although ATP secretion was partially inhibited and thromboxane production was inhibited totally by the indomethacin. Apyrase at 5 U/mL (based on 5′-ATPase activity), a concentration that effectively blocks irreversible aggregation induced by ADP, gave only slight inhibition of AG-1–induced aggregation.

The aggregation response of AG-1–treated platelets was inhibited by 5 mmol/L EDTA. Typically, the addition of AG-1 to a suspension of stirring platelets in the presence of EDTA resulted in a decrease in light transmittance after a significant lag phase. Examination of the platelets by scanning electron microscopy before and three minutes after the addition of AG-1 confirmed that the observed decrease in light transmittance was the result of platelet shape change, with prominent pseudopod development (data not shown).

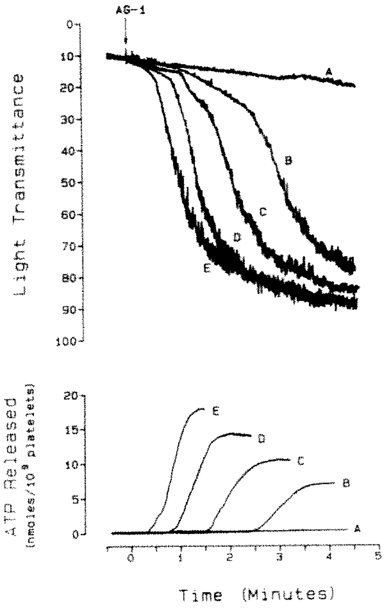

Fig 1. Induction of platelet aggregation and ATP secretion by AG-1. Citrated PRP (2×10^8 platelets per milliliter) was stirred at 1,200 rpm, 37 °C in the presence of 4 mg/mL luciferin-luciferase. Aggregation was monitored as increased light transmittance and ATP secretion as increased luminescence. AG-1 antibody was added at the arrow to a final concentration of (A) 0.78 μg/mL, (B) 1.56 μg/mL, (C) 3.13 μg/mL, (D) 6.25 μg/mL, or (E) 25.0 μg/mL.

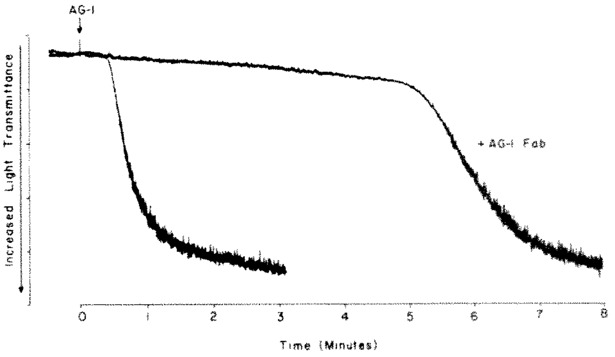

Fig 2. Effect of AG-1 Fab on platelet aggregation induced by intact AG-1. Citrated PRP (2.2×10^8 platelets per milliliter) was stirred at 37 °C for two minutes in the presence or absence of 10 μg/mL AG-1 Fab. At the arrow, intact AG-1 antibody was added to a final concentration of 12.5 μg/mL, and aggregation subsequently monitored.

Fab fragments prepared from AG-1 antibody failed to induce platelet aggregation. These monovalent antibody fragments did, however, inhibit the response of platelets to intact AG-1 antibody (Fig 2). At lower ratios of AG-1 Fab to whole antibody the aggregation response was delayed, while higher ratios resulted in complete inhibition of aggregation. In contrast, AG-1 Fab fragments did not inhibit platelet aggregation induced by ADP (8 μmol/L), epinephrine (8 μmol/L), collagen (8 μg/mL), γ-thrombin (120 nmol/L), or calcium ionophore A23187 (25 μmol/L).

The addition of AG-1 to the PRP of a patient with Glanzmann's thrombasthenia produced shape change and a normal amount of ATP secretion, but only a slight degree of aggregation—a pattern of responses identical to that seen in the patient with collagen or thrombin, suggesting a normal initial interaction of agonist with the platelets, but subsequent reduction in aggregation mediated by the platelet's fibrinogen receptors (which are known to be decreased in this disorder). Furthermore, preincubation of normal PRP with monoclonal antibody directed against the GPIIb/IIIa complex also blocked AG-1–induced aggregation (Fig 3). Albumin density gradient-washed platelets did aggregate in response to AG-1 in the absence of fibrinogen; however, this

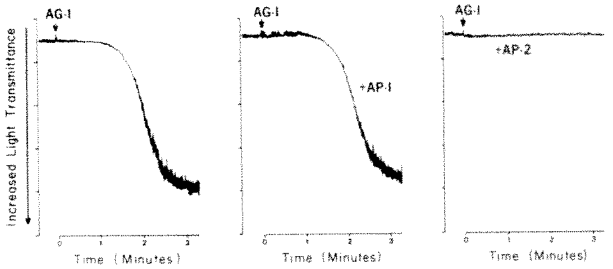

Fig 3. Effects of antibodies against GPIb and GPIIb/IIIa on platelet aggregation induced by AG-1. Albumin density gradient-washed platelets (2×10^8 platelets per milliliter) in Tyrode's buffer containing 1 mmol/L $CaCl_2$ and 50 μg/mL Kabi fibrinogen were stirred at 1,200 rpm at 37 °C. Platelets were preincubated with either buffer, 28 μg/mL AP-1 (anti-GPIb), or 24 μg/mL AP-2 (anti-GPIIb/IIIa) for one minute before the addition of AG-1 at a final concentration of 30 μg/mL.

Table 1. Effect of Platelet Function Inhibitors on [^{14}C]5-Hydroxytryptamine Secretion Induced by AG-1 or Thrombin

Condition	[^{14}C]5-Hydroxytryptamine Secreted (Percentage of Cell Content)	
	AG-1	Thrombin
Buffer	80.5 ± 3.9(4)	78.2 ± 6.0(4)
Dibutyryl cAMP	0.5 (2)	3.5 (2)
EDTA	9.7 ± 5.6(4)	84.3 ± 5.2(4)
Anti-GPIIb/IIIa	2.0 (2)	81.5 (2)
Anti-GPIb	80.0 (2)	86.5 (2)
Indomethacin	16.6 ± 7.8(3)	85.0 ± 5.3(3)
Adenosine	60.0 (2)	83.0 (2)

After uptake of [^{14}C]5-hydroxytryptamine by platelets, aliquots of washed platelets (1 × 10^8/mL) were incubated at 37 °C for ten minutes in the presence of buffer alone, 2 mmol/L dibutyryl cAMP, 5 mmol/L EDTA, 20 μg/mL AP-2 (anti-GPIIb/IIIa), 20 μg/mL AP-1 (anti-GPIb), 30 μmol/L indomethacin, or 1 mmol/L adenosine before the addition of AG-1 (20 μg/mL) or thrombin (1 U/mL). Incubation was continued under nonstirred conditions for another ten minutes before the release reaction was stopped by the addition of formaldehyde to a final concentration of 1.5% (wt/vol). Aliquots of platelet-free supernatant were counted in a scintillation counter. Results are expressed as means ± SEM, with the number of experiments in parentheses.

response was accelerated when fibrinogen was added (data not shown). Antibody directed against GP-Ib, which blocks vWF-mediated platelet agglutination induced by ristocetin, had no effect on AG-1–induced platelet aggregation (Fig 3).

Platelet-dense granule secretion induced by AG-1 was further studied by examining the secretion of [^{14}C]5-hydroxytryptamine from platelets. The results in Table 1 show that secretion induced by AG-1 occurred under nonstirred conditions and was inhibited by 5 mmol/L EDTA, 30 μmol/L indomethacin, 20 μg/mL AP-2, and 2 mmol/L dibutyryl cAMP. This contrasts with the [^{14}C]5-hydroxytryptamine secretion response induced by 1 U/mL thrombin, which was inhibited only by dibutyryl cAMP.

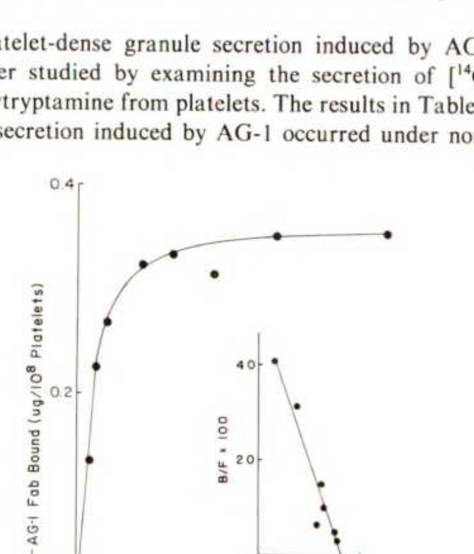

Fig 4. Specific binding of ^{125}I-labeled AG-1 Fab to platelets. Citrated PRP with a platelet concentration of 1 × 10^8/mL was mixed with increasing concentrations of labeled AG-1 Fab and incubated at 22 °C for 30 minutes without stirring. Platelet-associated radioactivity was determined after centrifugation of the platelets through a layer of 20% sucrose and counting of the platelet pellet. Nonspecific binding was determined in the presence of 800 μg/mL AG-1, and this value was subtracted from total binding, to yield specific binding. The inset is a Scatchard analysis of the binding data, from which the number of Fab binding sites was determined.

Binding of AG-1 Fab fragment to platelets. In order to estimate the number of sites recognized by AG-1 on platelets, binding studies were performed using ^{125}I-iodinated AG-1 Fab that had been prepared by pepsin digestion. Figure 4 shows a representative saturation binding curve and the inset, a Scatchard analysis of the binding data from which the average number of binding sites per platelet was derived. Nonspecific binding assessed in the presence of excess unlabeled AG-1 never exceeded 8% of the total counts bound. Based on the analysis of seven normal human subjects, there were approximately 65,000 AG-1 Fab binding sites per platelet.

Electrophoretic characterization of the AG-1 antigen. NP-40 lysates of ^{125}I-iodinated platelets were immunoprecipitated with monoclonal antibody AP-1 (directed against GPIb), AP-2 (directed against GPIIb/IIIa), or AG-1 and analyzed by 10% SDS-PAGE. On short exposures

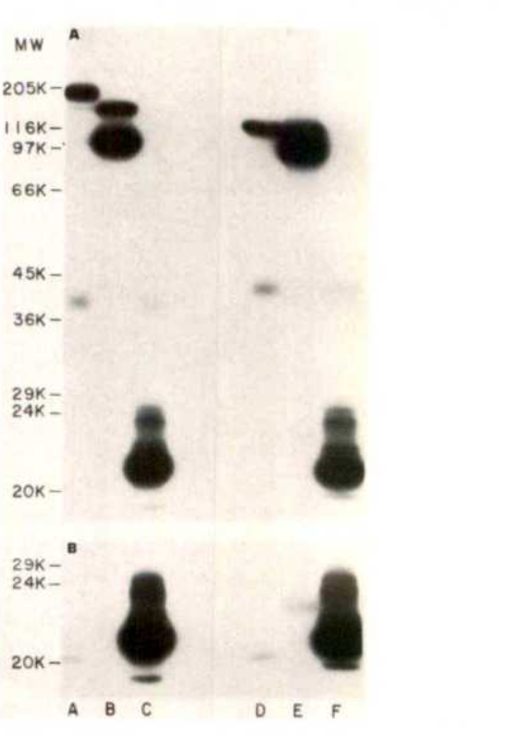

Fig 5. SDS-PAGE of AG-1, AP-1, and AP-2 immunoprecipitates of ^{125}I-labeled platelets. NP-40 lysates of ^{125}I-iodinated platelets were immunoprecipitated with 40 μg of AG-1, AP-1 (anti-GPIb), or AP-2 (anti-GPIIb/IIIa). Immunoprecipitates were analyzed by electrophoresis on 10% polyacrylamide gels, and polypeptides visualized by autoradiography. (A) Lane A, nonreduced AP-1; lane B, nonreduced AP-2; lane C, nonreduced AG-1; lane D, reduced AP-1; lane E, reduced AP-2, and lane F, reduced AG-1. (B) An autoradiograph of the same gel but a longer exposure to enhance the image of the GPIIb β-chain of ~23,000 daltons in lane E. (Mol wt markers: myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, and soybean trypsin inhibitor.)

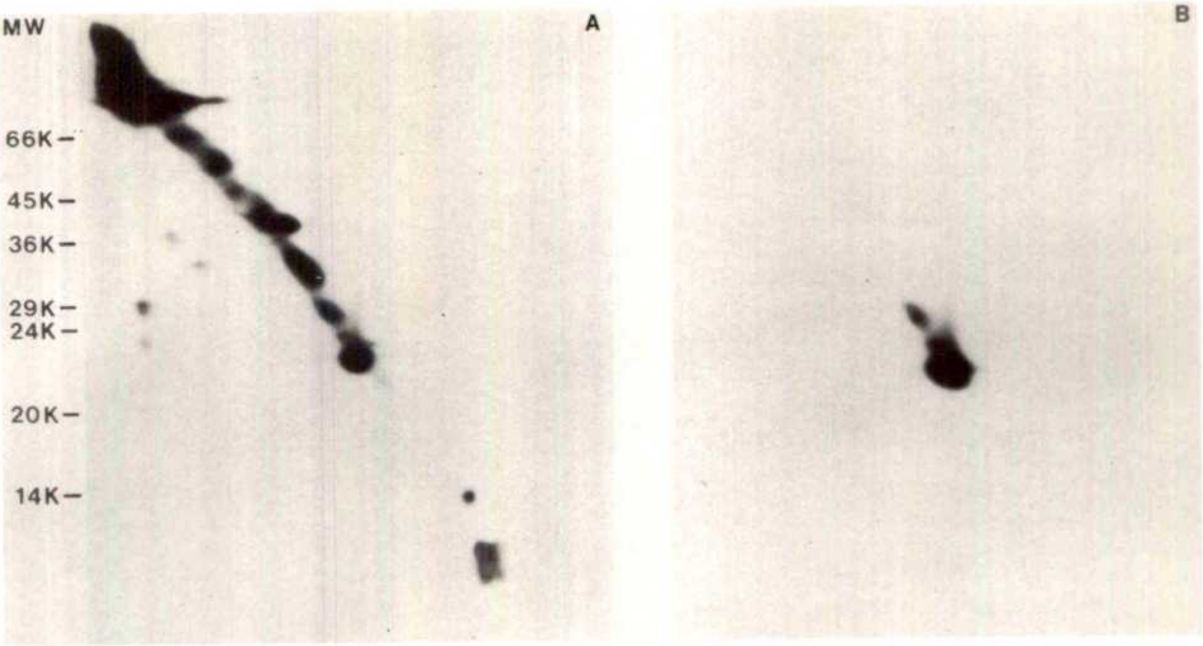

Fig 6. Two-dimensional nonreduced-reduced SDS-PAGE of ^{125}I-labeled platelets and AG-1 immunoprecipitate. Whole lysate of ^{125}I-iodinated platelets (A) and an AG-1 immunoprecipitate of ^{125}I-iodinated platelets (B) were electrophoresed nonreduced in a 12.5% polyacrylamide gel (left to right). After reduction with 2% β-mercaptoethanol, the polypeptides were separated in the second dimension on a 12.5% polyacrylamide slab gel (top to bottom). Labeled polypeptides were identified by autoradiography.

of the gels to x-ray film, the AG-1 immunoprecipitates run under either nonreduced or reduced conditions revealed only a single band of approximately 21,000 daltons. Longer gel exposures revealed a series of at least four additional bands in the 22,000- to 28,000-dalton range (Fig 5A, lanes C and F). AG-1 immunoprecipitates of the ^{125}I-iodinated platelets did not reveal bands in the positions of GPIb (Fig 5A, lanes A and D) or of GPIIb/IIIa (Fig 5A, lanes B and E). On still longer exposure of the same gel, a 23,000-dalton polypeptide in the reduced GPIIb/IIIa immunoprecipitate became visible (Fig 5B, lane E). This band representing the β-chain of GPIIb (Jennings et al)[25] is clearly distinct from the AG-1 immunoprecipitate. Additional studies by two-dimensional nonreduced-reduced SDS-PAGE of ^{125}I-labeled whole platelet lysates (Fig 6, panel A) and of AG-1–immunoprecipitated lysates (Fig 6, panel B) confirmed that the series of low-mol-wt polypeptides identified by AG-1 are in fact the major iodinated platelet membrane glycoproteins in this mol wt range. In addition, the appearance below the diagonal of at least the 21,000-dalton polypeptide recognized by AG-1 indicates a slightly increased mobility on reduction, suggesting the likelihood of intrachain disulfide bonds in this molecule.

The identity of the epitope recognized by AG-1 was studied further by the Western blot technique. Lane A of Fig 7 is an autoradiograph of an ^{125}I-labeled AG-1 immunoprecipitate after SDS-PAGE and electrophoretic transfer to nitrocellulose. The major 21,000-dalton band, as well as the series of higher bands, was seen to transfer well to the nitrocellulose. When an unlabeled whole platelet lysate was similarly electrophoresed and transferred to the nitrocellulose, immunoperoxidase staining for epitopes recognized by

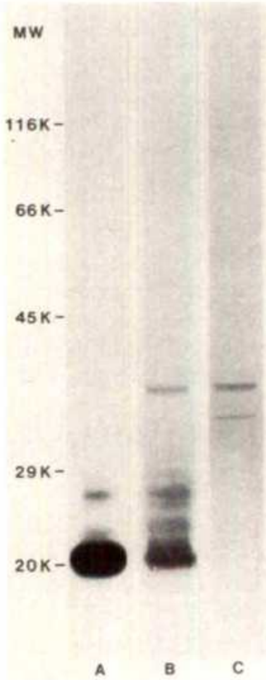

Fig 7. Western blot identification of AG-1 epitopes in platelet membrane proteins separated by 10% SDS-PAGE. Lane A, autoradiograph of an AG-1 immunoprecipitate of ^{125}I-labeled platelets separated by nonreduced 10% SDS-PAGE and then electroblotted onto nitrocellulose. Lanes B and C are from the same slab gel and represent AG-1 Western blots of platelet membrane proteins electrophoresed under nonreduced (lane B) and reduced (lane C) conditions.

AG-1 revealed an identical distribution (lane B). These bands were not detected if the platelet lysate was reduced before SDS-PAGE (lane C), or when blot studies of nonreduced platelet lysates were performed with isotypic control monoclonal antibodies substituted for AG-1. Two other bands located at approximately 35,000 daltons stained with AG-1 under both nonreduced and reduced conditions but were also stained with a monoclonal antibody to GPIb (data not shown), indicating that these bands stained nonspecifically. Those studies thus indicated that each of the low-mol-wt bands identified in the AG-1 immunoprecipitates of ^{125}I-labeled platelets represents a polypeptide containing the epitope recognized by AG-1, rather than membrane components loosely attached to or coprecipitating with a single polypeptide of uniform mol wt.

The major 21,000-dalton band seen in AG-1 immunoprecipitates of ^{125}I-labeled platelets was seen only faintly when AG-1 was used to immunoprecipitate platelets labeled by the periodate-[^{3}H]borohydride technique (Fig 8, lane B), suggesting a relative absence of carbohydrate containing sialic acid. Bands in the 22,000 to 28,000 region were more prominent, reflecting a greater degree of glycosylation of these polypeptides. In both the nonreduced (lane B) and reduced (lane F) AG-1 immunoprecipitates of ^{3}H-labeled platelets, bands were present that comigrated with GPIb immunoprecipitated by monoclonal antibody AP-1 (lanes A and E). The 17,000-dalton band (GPIX) coprecipitating with GPIb in AP-1 immunoprecipitates (lane A) was also seen faintly in lane B, suggesting that GPIX (as well as GPIb) was in fact present in the AG-1 immunoprecipitates. After reduction of the AP-1 immunoprecipitate, the β-chain of GPIb could be identified at approximately 22,000 daltons and GPIX at approximately 20,000 daltons (lane E). AG-1 immunoprecipitates of ^{125}I-labeled platelets run alongside AP-1 immunoprecipitates of ^{3}H-labeled platelets under reducing conditions showed migration of these bands to be distinct from the major 21,000 band or the 22,000 to 28,000 bands recognized by AG-1 (data not shown).

The results of the above experiments suggested the presence of GPIb in AG-1 immunoprecipitates. However, precipitation of equal volumes of ^{3}H-labeled platelet lysates with AG-1 or with the anti-GPIb antibody AP-1 resulted in 20 times more ^{3}H in the AP-1 immunoprecipitate. This indicates that only a small proportion ($\sim$5%) of the GPIb recognized by AP-1 is found associated in AG-1 immunoprecipitates.

In order to explore further the possible relationships between GPIb and the AG-1 epitope suggested by the preceding studies, we performed immunoprecipitation of ^{3}H-labeled platelets with AP-1 or with AG-1, followed by precipitation of the residual supernatant with the other antibody. After initial immunoprecipitation of the platelet lysate with AG-1, subsequent immunoprecipitation of the "precleared" lysate with AP-1 (Fig 8, lanes C and G) yielded bands indistinguishable from those resulting from AP-1 immunoprecipitation alone. Of considerable interest was the repeated finding that when AP-1 was used to "preclear" the platelet lysate, a subsequent AG-1 immunoprecipitate (Fig 8, lane D) was virtually identical to the direct AG-1 immunoprecipitate, except for absence of a single band—the high-mol-wt band migrating above the GPIb bands immunoprecipitated by AP-1. When the "precleared" immunoprecipitate was run under reducing conditions (Fig 8, lane H), the band pattern did not appear significantly different from that resulting from AG-1 immunoprecipitation alone (Fig 8, lane F).

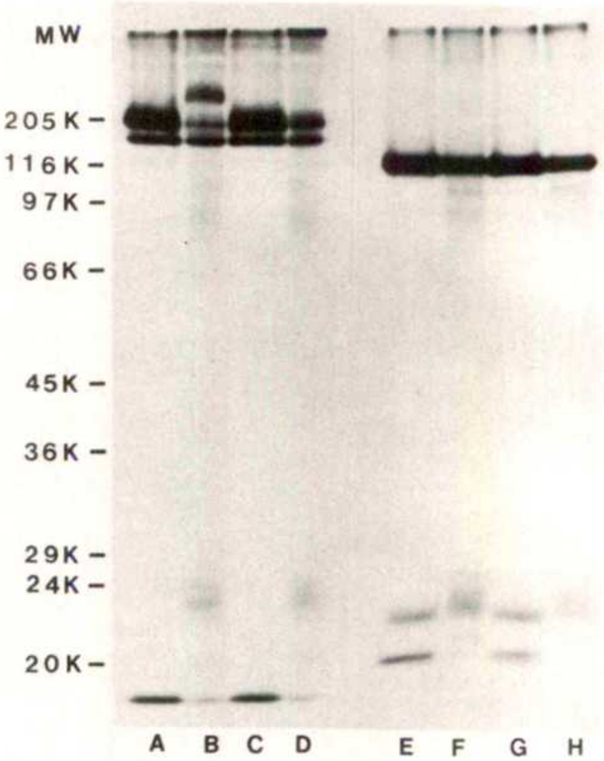

Fig 8. SDS-PAGE of immunoprecipitations of [^{3}H]-labeled platelets by monoclonal antibodies AP-1 and AG-1. Platelets labeled by the periodate-[^{3}H]borohydride technique were lysed with NP-40, immunoprecipitated with AP-1 or AG-1, and analyzed by 10% SDS-PAGE. Lanes A and E, AP-1 immunoprecipitates (nonreduced and reduced, respectively). Lanes B and F, AG-1 immunoprecipitates (nonreduced and reduced, respectively). After immunoprecipitation with either AP-1 or AG-1, the residual "precleared" platelet lysate was then immunoprecipitated by the other monoclonal antibody. Lanes C and G, AP-1 immunoprecipitates of lysates precleared by AG-1 (nonreduced and reduced, respectively); lanes D and H, AG-1 immunoprecipitates of lysates precleared by AP-1 (nonreduced and reduced, respectively). Although AG-1 immunoprecipitates contained only about 5% as many cpm as AP-1 immunoprecipitates, aliquots were diluted to allow the application of equal numbers of counts to each lane.

DISCUSSION

We have described platelet activation induced by a monoclonal antibody against human platelets and have characterized the membrane protein recognized by this antibody. Activation was initiated by a defined molecular interaction at the cell surface, the binding of antibody AG-1 to its membrane epitopes. Activation was not mediated by the Fc region of the antibody, as indicated by the capacity of Fab fragment to block platelet aggregation induced by intact AG-1 antibody. Aggregometry and scanning electron microscopy showed that AG-1 was capable of inducing platelet shape change in the presence of EDTA without appreciable agglutination. Incubation of platelets with AG-1 resulted in a strong thromboxane response even under nonstirred conditions. These results suggest that AG-1–induced activation was not the result of passive, antibody-mediated platelet agglutination.

AG-1 Fab fragments inhibited platelet aggregation induced by low concentrations of whole AG-1 antibody and delayed the onset of aggregation induced by higher antibody concentrations, a finding that is consistent with a requirement for cross-linking of the membrane antigen recognized by AG-1. The failure of the AG-1 Fab fragment to fully inhibit aggregation induced by high AG-1 concentrations probably reflects dissociation of the Fab fragments by and increased membrane binding of the intact AG-1.

A strong dense granule secretion response was induced by AG-1 under nonstirred conditions. Whereas [^{14}C]5-hydroxytryptamine secretion was only partially blocked by indomethacin, secretion was strongly inhibited by EDTA or by anti-GPIIb/IIIa monoclonal antibody. In contrast, EDTA and anti-GPIIb/IIIa antibody had no effect on thrombin-induced secretion. Recently, Powling and Hardisty[26] have shown that an anti-IIb/IIIa monoclonal antibody (M148) could significantly inhibit Ca^{++} influx and ATP secretion by platelets stimulated with ADP, platelet-activating factor, or low thrombin concentrations. They proposed the existence of a Ca^{++} channel adjacent to the GPIIb/IIIa complex that could be inhibited by anti-GPIIb/IIIa antibodies. Another interpretation is that ATP secretion induced by weak agonists, including AG-1, is aggregation dependent and that even under nonstirred conditions, fibrinogen-dependent microaggregate formation occurs and potentiates secretion.

That AG-1 platelet activation may be under the control of intracellular cAMP is suggested by the inhibitory effect of dibutyryl cAMP on AG-1–induced 5-hydroxytryptamine secretion. The potentiation of the aggregation response in the washed platelet system on the addition of fibrinogen, diminished aggregation response of thrombasthenic platelets to AG-1, and the inhibitory effect of an anti-GPIIb/IIIa monoclonal antibody (AP-2) on normal platelets suggest that AG-1–induced aggregation is in fact dependent on fibrinogen binding to GPIIb/IIIa.

Heterologous antihuman platelet antiserum has been shown to cause in vitro platelet aggregation, ATP and 5-hydroxytryptamine release, and prostaglandin formation by a calcium-dependent mechanism that was not complement-mediated.[27-29] The aggregation and secretion responses in these studies were not effectively inhibited by aspirin, suggesting an activation process different from that induced by ADP and epinephrine. These characteristics of the heterologous antiplatelet antibody-induced activation are similar to the AG-1–induced response described. However, the multispecificity of a heteroantiserum to whole cells makes this platelet activation response and its analysis complex.

Several groups have reported monoclonal antibodies against low-mol-wt membrane proteins of platelets that are capable of inducing platelet aggregation and secretion. Thiagarajan et al[30] briefly described an antibody to a 21,000-dalton polypeptide that could induce human platelet aggregation inhibitable by EDTA. Boucheix et al[31] described a monoclonal antibody, ALB_6, prepared against human leukemic cells that could induce platelet aggregation, and Enouf et al[32] showed that ALB_6 could induce calcium uptake by platelet membrane vesicles. The latter study showed that the 24,000-dalton protein recognized by ALB_6 was not identical to the low-mol-wt platelet protein involved in calcium transport that is phosphorylated by a cAMP-dependent protein kinase. During the course of the present study, Higashihara et al[33] reported on a monoclonal antibody to a 23,000-dalton platelet polypeptide that induced platelet aggregation and ATP secretion under stirring conditions. This antibody was also reported to block ristocetin-induced platelet aggregation. Jennings et al[25] have reported that several monoclonal antibodies against the β-chain of GPIIb induced platelet activation, and that Fab fragments of these antibodies were as effective as the whole antibody molecule.

We have shown that AG-1 recognizes platelet membrane polypeptides that are distinct from the β-chains of either GPIb or GPIIb, as well from the 17,000-dalton (reduced) polypeptide associated noncovalently with GPIb[34] that has been identified by some as GPIX.[35] The major AG-1–reactive polypeptide of approximately 21,000 daltons appears to be related to at least four larger, cross-reactive polypeptides that may represent glycosylation variants. These polypeptides appear to possess at least one intrachain disulfide bond, as suggested by the slightly increased mobility of the 21,000-dalton chain under the reducing conditions of two-dimensional nonreduced-reduced SDS-PAGE. The presence of such disulfide bonds is also suggested by the lability of the AG-1 epitope under reducing conditions, as demonstrated by Western blotting. Newman et al[36] have characterized a 24,000-dalton polypeptide found on a variety of acute lymphocytic leukemia cells and on some chronic lymphocytic leukemia cells. This protein (p24/BA-2) was glycosylated and had a pI near 7, which is similar to the polypeptide recognized by AG-1 (data not shown). However, the 24,000-dalton polypeptide described by Newman et al appeared to lack intrachain disulfide bonds. In addition, immunoprecipitation experiments failed to detect any molecular associations between this polypeptide and other membrane proteins of the leukemic cells.

In the periodate-[^{3}H]borohydride–labeled AG-1 immunoprecipitates, bands were identified that comigrated with GPIb. The likelihood that this material does actually represent GPIb is further strengthened by the finding that the 17,000-dalton band (GPIX) coprecipitating with GPIb in the ^{3}H-labeled AP-1 immunoprecipitates was identically observed in the AG-1 immunoprecipitates. In addition, a band migrating with a higher mol wt than those identified in the AP-1 immunoprecipitates was observed. Using wheat germ lectin affinity chromatography, Nachman et al[37] described a 210,000-dalton protein that these authors believed to be a structural analogue of GPIb. Kunicki et al[38] also demonstrated in the eluate from a wheat germ agglutinin column a fraction containing a high-mol-wt (190,000- to 210,000-dalton) glycoprotein. In both of these studies, the relative mobility in SDS-PAGE gels of the high-mol-wt glycoprotein was not significantly affected by disulfide bond reduction. Although the implications of these findings remain unresolved, the highest-mol-wt band detected in the nonreduced AG-1 immunoprecipitates in the present study appears unlikely to represent the same structure. For, after reduction, this band disappears entirely, and only a single band with a mol wt identical to the GPIb alpha chain is seen in this region.

An intriguing finding in the present studies was that when ^{3}H-labeled platelet lysates were first immunoprecipitated with AP-1, and these "precleared" lysates were subsequently immunoprecipitated with AG-1, the nonreduced AG-1 immunoprecipitates no longer revealed the highest-mol-wt bands. Removal of this band by AP-1 appeared selective, since AG-1 continued to precipitate ^{3}H-labeled protein comigrating with the GPIb bands seen in direct AP-1 immunoprecipitates. These results raise the interesting possibility that there may exist a previously unrecognized heterogeneity of GPIb molecules. The ability of AP-1 to remove the highest-mol-wt band suggests that this material does represent a form of GPIb. Those GPIb molecules persisting after preclearing by AP-1 may be complexed with the low-mol-wt peptides in such a way as to mask the AP-1 recognition site. Those GPIb molecules, however, that are associated with the highest-mol-wt band might possess a variant structure, such as variability in disulfide bonding pattern, that permits association with the low-mol-wt peptides recognized by AG-1, while still retaining exposure of the AP-1 recognition site.

Although our findings suggest that macromolecular associations may exist between GPIb and polypeptides recognized by AG-1, under the conditions of these studies only a relatively small percentage (approximately 5%) of the total pool of ^{3}H-labeled GPIb recognized by AP-1 would actually appear to associate with the low-mol-wt polypeptides. Of this already small percentage, it is only those GPIb molecules that are actually precleared by AP-1 from the AG-1 immunoprecipitates that would be expected to be associated with the low-mol-wt polypeptides (ie, only 1% to 2% of the total GPIb recognized by AP-1). The absence of a significant 21,000-dalton band in AP-1 immunoprecipitates of ^{125}I-labeled platelets, despite the relatively high labeling efficiency of this polypeptide for iodine, suggests that there may in fact not be a high degree of association of this polypeptide with GPIb. In contrast, GPIb may preferentially associate with the 22,000- to 28,000-dalton polypeptides that also bear the AG-1 epitope. Such a possibility would be consistent with the absence of low-mol-wt bands in AP-1 immunoprecipitates of ^{125}I-labeled platelets, since these latter polypeptides label much less efficiently with iodine than does the major 21,000-dalton polypeptide. In view of the ability of monoclonal antibody AG-1 to induce platelet activation, further study of the molecular associations of GPIb with the series of low-mol-wt polypeptides recognized by AG-1 may well provide additional insights into platelet function.

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Monoclonal Antibody T101 in T Cell Malignancies: A Clinical, Pharmacokinetic, and Immunologic Correlation

By Juergen H. Bertram, Parkash S. Gill, Alexandra M. Levine, Daisy Boquiren, Florence M. Hoffman, Paul Meyer, and Malcolm S. Mitchell

Eight patients with cutaneous T cell lymphomas (CTCL) and five with various other T cell malignancies were treated with mouse monoclonal antibody (MoAb) T101. Doses of 1 to 500 mg were administered weekly over a two-hour period and resulted in one complete remission (convoluted T cell lymphoma) and one partial remission (CTCL). Remission duration was 6 weeks and 3 months, respectively. Frequent toxicities were pruritus, hives, flushing, and shortness of breath. Supraventricular arrhythmias and blood pressure instability were also observed. Complete targeting of peripheral blood T cells was achieved with 1 mg of MoAb in the nonleukemic patients (WBC $< 10{,}000/\mu L$), and free, bioavailable antibody was present at the next (10-mg) dose level. Even higher doses resulted in substantial antibody excess that persisted for as long as 6 weeks. Serum concentrations of MoAb decreased with increasing number of peripheral blood T cells, and 25 to 35 mg of T101 were required for induction of antibody excess in leukemic patients. Excess antibody induced antigenic modulation, which was of consequence only if MoAb excess persisted to the next treatment. In the original treatment, the rapidly administered MoAb was able to target and remove peripheral blood T cells before the development of antigenic modulation. Antimouse antibodies developed in three patients. Their presence rendered further therapy ineffective and was associated with an anaphylactic reaction in one patient. Development of these antibodies could not be predicted by lymphoproliferative assays. In these assays, however, the T101 protein strongly stimulated the mononuclear cells of the patient who reached the only complete remission of this trial. Immunologic stimulation by the MoAb thus might have played a role in this patient's antitumor response. In summary, therapy with MoAb T101 was specific but only modestly efficacious. Rapid infusion of nonmodulating doses of antibody provided excellent targeting and removal of peripheral blood T cells and might be a valid approach in future trials with immunoconjugated T101.

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T CELL MALIGNANCIES are a heterogeneous group of diseases. They include the cutaneous T cell lymphomas, T cell–derived acute lymphoblastic leukemia (T-ALL), and subsets of poorly differentiated lymphocytic lymphoma and histiocytic lymphoma. These last two entities are now known as convoluted T cell lymphoma and T cell immunoblastic sarcoma, respectively.[1,2] Cells from many of these T cell disorders express a 65,000-dalton antigen (T65) that is a normal T cell differentiation antigen.[3] The monoclonal antibodies (MoAb) T101, L17F12, and anti–Leu-1 are directed against this T65 antigen[4] and were used clinically in a small number of patients with various T cell malignancies.[5-8] They were also used in B cell–derived chronic lymphocytic leukemia (CLL),[8-10] which also expresses the T65 antigen.[11] Encouraging antitumor activity was noted in some of these patients. Antigenic modulation, however, severely interfered with MoAb-induced peripheral blood T cell removal[9,10,12] and was particularly severe with prolonged (24-hour or longer) infusion schedules.

We present here our experience with the MoAb T101 in patients with T cell malignancies. A two-hour infusion schedule was adopted for this trial in an attempt to minimize antigenic modulation. Toxicity, in vivo pharmacokinetics, and antitumor activity of this short-term MoAb infusion are reported. Factors preventing interaction of MoAb with its target such as formation of antimouse antibodies, therapy-induced liberation of cell surface antigens, and antigenic modulation were also carefully determined. These findings were correlated with observed degree of peripheral blood T cell removal and serum concentrations of T101 MoAb. Lymphoproliferative assays were also performed serially during and after each treatment in all patients to examine the potential influence of MoAb on this parameter of host immunity.

MATERIALS AND METHODS

Patients. Patients with histologically confirmed T cell malignancies (12 patients) or B cell CLL (one patient) were enrolled in this study. Important clinical and laboratory characteristics are given in Table 1. Almost all patients were heavily pretreated and were refractory to standard therapies. Patients were staged prior to enrollment in the study with computerized tomography (CT) of the chest, abdomen, and pelvis. Bone marrow aspiration and biopsies were also performed in all patients prior to the beginning of MoAb therapy, and skin biopsy specimens were obtained from the CTCL patients. None of the patients had received chemotherapy or radiotherapy within 4 weeks of the beginning of this trial or during antibody therapy. Patients evaluable for response also received no therapy for 4 weeks after completion of MoAb therapy. Informed consent was obtained from all patients according to institutional and National Cancer Institute (NCI) policies.

Antibody preparation and administration. MoAb T101 produced by Hybritech Inc (San Diego) and provided by NCI was administered intravenously for a two-hour period. Treatments were given once weekly for a total of four doses. The initial antibody dose was 1 mg, and subsequent dose levels were 10, 50, 100, and 500 mg, each given to three previously untreated patients. Dose escalation within a given patient was also permitted provided there was no severe toxicity noted at the previous dose. Antibody doses were diluted to 500 mL with normal saline and were administered with continuous-flow monitoring.

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Table 1. Clinical and Laboratory Characteristics of 13 Patients Treated With MoAb T101

Patient No.	Age/Sex	Diagnosis	Previous Treatment	Site of Disease	No. of Treatments	T101 (mg)	WBC $\times 10^3/\mu L$	Percentage of Ly	Percentage of T101	Antigenic Modulation (During Treatment No.)	Antimouse Antibodies	Clinical Response
1	26/F	Convoluted T cell	Ara-C, 6-TG, VCR, L-ASP, MTX, 6-MP	BM	4	1–100	5,800	29	61	Yes (2–4)	Yes	CR
2	73/M	CTCL	Prednisone, RT, *N*-mustard	Skin, BM	4	1–100	7,500	24	25	Yes (2–4)	No	NR
3	74/M	CTCL	MOPP, *N*-mustard ATG, RT	Skin, BM	3	1–50	5,500	34	27	Yes (2–3)	No	NR
4	28/M	T-ALL	Ara-C, 6-TG, VCR, MTX, Adriamycin, Cytoxan, MOPP, CVP	BM, PB	1	10	700	95	95	—	No	NE
5	62/M	CTCL	MOPP, CVP, RT, ATG	Skin	4	10–500	5,200	23	18	Yes (2–4)	No	PR
6	78/F	B-CLL	CVP, cytoxan, VCR, Adriamycin, BLEO, MTX, prednisone	BM, PB	4	10–500	34,600	71	65	Yes (2–4)	No	NR
7	60/F	CTCL	CHOP, MOPP, RT, prednisone	Skin	3	50–500	9,900	7	63	Yes (2–3)	No	PD
8	72/F	T-IBS	Ara-C, 6-TG, VCR Mtx, CVP	BM, skin, spleen	1	50	2,200	12	71	Yes	No	NE
9	50/M	CTCL	Topical steroids	Skin	3	50–500	20,200	6	74	Yes (1–2)	Yes	NR
10	40/M	CTCL	Topical steroids, RT	Skin	4	50–500	3,900	18	71	Yes (1–4)	No	NR
11	36/F	CTCL	MOPP, *N*-mustard, Adriamycin, BLEO, RT	Skin	4	100–500	7,100	11	87	Yes (1–3)	Yes	NR
12	56/M	T-CLL	CVP, splenectomy	BM, PB	2	25, 35	35,600	87	84	No	No	NR
13	57/F	Sézary syndrome	*N*-mustard, BLEO, RT	Skin, BM, PB	2	25, 25	128,000	95	95	Yes (2)	No	NR

Abbreviations: CTCL, cutaneous T cell lymphoma; T-IBS, immunoblastic T cell sarcoma; BM, bone marrow; PB, peripheral blood; NR, no response; PR, partial remission; CR, complete remission; PD, progressive disease; NE, not evaluable; Ara-C, cytosine arabinoside; L-ASP, L-asparaginase; BLEO, bleomycin; 6-MP, 6-mercaptopurine; MTX, methotrexate; 6-TG, 6-thioguanine; CHOP, cytoxan, Adriamycin, vincristine; CVP, cytoxan, vincristine, prednisone; MOPP, nitrogen, mustard, vincristine, procarbazine, prednisone; ATG, antithymocyte globulin; RT, electron beam irradiation; Ly, lymphocytes.

Table 2. Frequent Toxicities of T101 Therapy

Toxicity	Frequency* (%)
Pruritus	57
Hives	43
Increased BP	37
Sinus tachycardia	33
Flushing	27
Tachypnea	27
Decreased BP	27
Fever	23
Dyspnea	23
Chest pressure	23
Wheezing	20
Diaphoresis	16
Arrhythmias	16
Nausea	16
Vomiting	14
Abdominal cramps	14
Diarrhea	14

Abbreviation: BP, blood pressure.

*Frequency represents the ratio of the number of observed toxic episodes to the number of treatments administered.

Therapy was discontinued after administration of four MoAb doses regardless of the outcome of therapy. Tumor measurements were obtained weekly from patients with CTCL and in those with measurable peripheral disease. CT scanning and bone marrow biopsies were repeated in all patients at the end of therapy. Skin biopsies were repeated in patients with CTCL who had signs of antitumor response. Antitumor responses were classified according to the World Health Organization criteria. Patients who had obtained at least stabilization of disease were eligible for retreatment at the time of relapse.

Monitoring for toxicity. Vital signs were obtained every 15 minutes during the infusion period and then every 30 minutes for the next four hours. All patients were also evaluated by continuous electrocardiographic bedside monitoring. Complete blood counts (CBC), serum electrolyte determinations, and blood studies for determining renal and hepatic functions were performed weekly during the treatments and for 4 weeks after completion of therapy.

Monitoring for in vivo effects of T101 antibody. Blood for CBC and T cell subset determinations and sera for blocking assays, T101 serum concentration determinations, and endogenous antimouse antibody levels were obtained at 0, 1, 3, 5, 7, 12, 24, 48, 72, and 96 hours during initial treatment. On subsequent treatments, the seven- and 12-hour blood specimens were omitted. Bone marrow aspirations were obtained immediately prior to and 24 hours after MoAb administration in three patients. Skin biopsies performed on two patients with CTCL were obtained at four and 24 hours after the onset of therapy.

Immunofluorescence assays. These assays were performed immediately after therapy on whole blood specimens and again after completion of this trial simultaneously on cryopreserved cells of all patients. One hundred microliters of whole blood was diluted 1:1 with a lysing solution containing MgCl (Ortho Diagnostics, Westwood, Mass), and after ten minutes, 10 μL of the MoAb T3, T4, T8, T11 (Ortho) or 10 μL of a 1:100-diluted T101 MoAb was added. Specimens were incubated for one hour at 4 °C and washed, and thereafter 20 μL of a 1:40-diluted fluorescein isothiocyanate (FITC)-conjugated goat antimouse F(ab')$_2$ fragments was added (Cappel Laboratories, Cochranville, Pa). After another one-hour incubation period at 4 °C, cells were washed again and resuspended in 1 mL of 0.1 mol/L phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) and sodium azide, and the resulting fluorescence was determined on a cytofluorograph 5 OH (Ortho). Purified mouse myeloma protein of the IgG2a subclass served as a negative control (RPC-5, Bethesda Research Laboratories, Rockville, Md). The immunofluorescence assays with cryopreserved cells were performed in an identical fashion except that 1×10^6 purified mononculear cells were used instead of whole blood. These cells were isolated by Ficoll-Hypaque gradient centrifugation and then frozen at a constant rate of 1 °C/min. Cells were stored in the vapor phase of liquid nitrogen.

In vivo binding of the T101 antibody after therapy was determined by the immediate addition of 20 μL of the 1:40-diluted FITC-goat antimouse F(ab')$_2$ fragment to 1×10^6 isolated mononuclear cells. The intensity presented for all measured fluorescence is the mean channel of fluorescence intensity of the Ortho cytofluorograph 5 OH.

Immunoperoxidase staining procedure. Fresh biopsy specimens were embedded in ornithine carbamyl transferase (OCT) (Miles Scientific, Naperville, Ill) and snap-frozen in a liquid nitrogen–isopentane mixture (−12 °C to −15 °C). These tissues were stored at −70 °C until sectioned. Cryostat sections were cut at 7 μm and allowed to air-dry overnight. Immediately before staining, the slides were fixed in acetone for ten minutes at 25 °C and then washed with PBS (pH 7.4) for three minutes. The MoAbs and secondary linking reagents used for staining were also prepared with this PBS solution. The tissue sections were incubated with the primary antibody for 30 minutes at 25 °C in a humidified chamber and then washed in PBS for ten minutes. The peroxidase-labeled secondary antibody (peroxidase-conjugated goat antimouse IgG, Tago Inc, Burlingame, Calif) was applied to the section for 30 minutes. The slides were washed, and the colored substrate aminoethyl carbazole (AEC, Sigma, St Louis) was applied to the tissue and incubated for ten minutes. The slides were then rinsed in tap water for five minutes, stained with Mayer's hematoxylin for ten minutes, and mounted with Aquamount (Lerner Labs, Sewickley, Pa). Controls included replacement of the primary antibody with a nonspecific antibody (ascites) and omission of the primary antibody altogether.

Determination of the serum concentration of T101 MoAb. Serum concentrations of T101 were determined in all patients by a cell-binding competitive inhibition radioimmunoassay that measures immunoreactive T101. This assay was performed by adding 50 μL of variously diluted sera to 5×10^5 of 8402 cells (a T lymphocytic leukemia cell line that expresses the T65 antigen in high concentrations) followed by overnight incubation at 4 °C. Cells were then washed and resuspended in 50 μL PBS containing 1% BSA, and 50 μL of iodinated T101 MoAb (1 to 2×10^6 cpm) was added. After an additional four-hour incubation period, cells were washed extensively and bound radioactivity determined. Serum concentrations of T101 MoAb were determined from a standard curve created with various concentrations of purified T101.

This cell-binding assay was accurate to approximately 400 ng of T101/mL. Above this concentration, the binding capacity of the T cells was exhausted, and a solid-phase radioimmunoassay was used concomitantly to minimize sequential dilution errors. This assay detected up to 6 μg of T101/mL and was performed by overnight coating of the wells of polyvinyl chloride microtiter plates with 10 μg/mL of purified goat antimouse Ig (Cappel). In the morning, plates were washed, and 50 μL of serially diluted sera was added and incubated for one hour. Thereafter the plates were washed again. Fifty microliters of ^{125}I-labeled T101 was added, plates were incubated and washed, and the amount of bound radioactivity was determined in a gamma counter. Various dilutions of T101 were used to create a standard curve for quantitative measurement. This solid-phase radioimmunoassay was not reliable in the presence of antimouse antibodies or other blocking serum activities and was principally used to confirm the measurements obtained by the cell-binding assay.

Assay for blocking activity in serum. To determine whether blocking activity, ie, the T65 antigen or antimouse antibodies, was present, 100 μL of patient serum was incubated with 100 μL of variously diluted T101 antibody (1:2, 1:5, 1:50) for one hour at 4 °C. Subsequently, 2×10^5 of 8402 cells was added and incubated for one hour at 4 °C. Cells were then washed and 50 μL of 1:40-diluted FITC-labeled goat antimouse antibody added (Cappel) and incubated for one hour at 4 °C. The resulting fluorescence was determined with a FACS III analyzer (Becton Dickinson, Mountain View, Calif) after cells were washed again with PBS containing 1% BSA and sodium azide. If blocking activity was present in the patient's serum, the assay for endogenous antimouse antibody was performed.

Assay for endogenous antimouse antibody. An immunoprecipitation method was used for these measurements. Five to fifty microliters of ^{125}I-T101 (1×10^6 cpm) was added to 50 μL of variously diluted sera and incubated for eight hours at 4 °C. Thereafter, 50 μL of goat antihuman Ig (Cappel) coupled to Sepharose 4B was added and incubated overnight at 4 °C. The resulting precipitate was then washed with PBS and counted in a gamma counter. Sera from patient 10, who had an anaphylactic reaction because of the presence of antimouse antibodies, served as the positive control in all assays.

Assay for lymphoproliferation. Mononuclear cells from normal individuals and all patients were prepared from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Cells of patients and normal individuals (alloantigen) were dispensed into microtiter wells at a concentration of 2×10^5/well each in 100 μL of RPMI 1640 medium. Twenty microliters of normal serum or patients' pretreatment or posttreatment sera was then added to the wells. In certain experiments, concanavalin A (20 μg/well) was used instead of the normal mononuclear cells. Plates were cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C. Wells were then pulsed with 1 μCi/well of ^{3}H-thymidine. Cells were harvested 18 hours later onto glass fiber strips, and thymidine uptake was measured by scintillation counting. Data are expressed as the means of quadruplicate wells.

RESULTS

Thirteen patients were entered into this trial, including eight patients with CTCL and one patient each with convoluted T cell lymphoma, T-ALL, T cell CLL, T cell immunoblastic sarcoma, and B cell CLL. Their clinical and laboratory characteristics are given in Table 1. All patients were evaluated for toxicity and efficacy. Doses of T101 MoAb ranged from 1 to 500 mg, and a total of 39 doses was administered.

Toxicity of T101 therapy. Toxic effects were noted in all patients during all treatments, and their frequence of occurrence is listed in Table 2. Pruritus, hives, flushing, and facial edema were the most common side effects of this trial and occurred at all dose levels. These toxicities were usually seen within 30 to 60 minutes after start of the infusion and regressed spontaneously within 24 hours. Mild to moderate dyspnea was observed in eight patients, requiring supplemental oxygen in all and the use of bronchodilators in one. This side effect usually developed one hour after the beginning of the antibody infusion and was most pronounced during each patient's first treatment regardless of the initial MoAb dose. Life-threatening anaphylaxis leading to study termination occurred in patient 9 with the third T101 MoAb dose (500 mg). Administration of this dose was delayed for 2 weeks because of patient noncompliance. Antimouse antibodies were present at that time and may have caused the anaphylactic reaction.

Blood pressure instability and cardiac arrhythmias occurred at or above the 10-mg dose level and prevented administration of the four MoAb doses in five patients. Rapidly changing blood pressures were noted in patients 3 and 13. Both individuals were normotensive prior to therapy but had blood pressure readings as high as 210/130 mm Hg in the course of therapy. Blood pressures began to rise approximately 30 to 60 minutes after the beginning of the antibody infusion. With continuation of therapy, blood pressures fluctuated widely, and readings as low as 90/50 mm Hg were observed. Blood pressure changes occurred in patient 3 at the 10-mg dose level and caused termination of therapy during the third (50 mg) treatment. In patient 13, antibody infusion had to be terminated prematurely in both attempted treatments.

Premature atrial contractions (PACs), which were observed in seven patients, were accompanied in all instances by sinus tachycardia and were clinically asymptomatic. They were especially frequent in patient 8. Study was terminated in this patient as a precautionary measure after one treatment.

In patient 12, who had no known preexisting cardiac disease and a normal on-study ECG, a second-degree atrioventricular block developed approximately one hour after the start of the first MoAb treatment (100 mg). The pulse rate decreased concomitantly from 130 to 80 beats/min. An identical arrhythmia occurred with the second MoAb dose, which had been reduced to 50 mg. With both treatments, normal sinus rhythm promptly ensued 60 minutes after termination of the infusion. This patient was subsequently withdrawn from the study. Atrial fibrillation developed in patient 6 during the first treatment (10 mg T101). The relationship of this toxicity to T101 therapy, however, was uncertain since this patient had previous episodes of intermittent atrial fibrillation. Study was continued, and cardiac side effects were absent in the subsequent three treatments.

Gastrointestinal toxicity including nausea, vomiting, diarrhea, and abdominal cramps was seen in a minority of patients. It occurred at doses of 10 mg or above.

The dose-limiting toxicity was reached with an initial T101 dose of 100 mg. At that dose level, all treated patients were pale, tachycardic, and profoundly diaphoretic. Interestingly, doses of 100 mg or higher (500 mg) could be delivered if therapy was begun at a lower dose level. This decrease of toxicity with dose escalation was most prominent in patients who either had persistent antigenic modulation or in whom antimouse antibodies were present.

In vitro targeting of MoAb T101 and presence of blocking serum factors prior to therapy. Before enrollment in this study, each patient's peripheral blood lymphocytes were isolated and reacted with MoAb T101. Most patients with

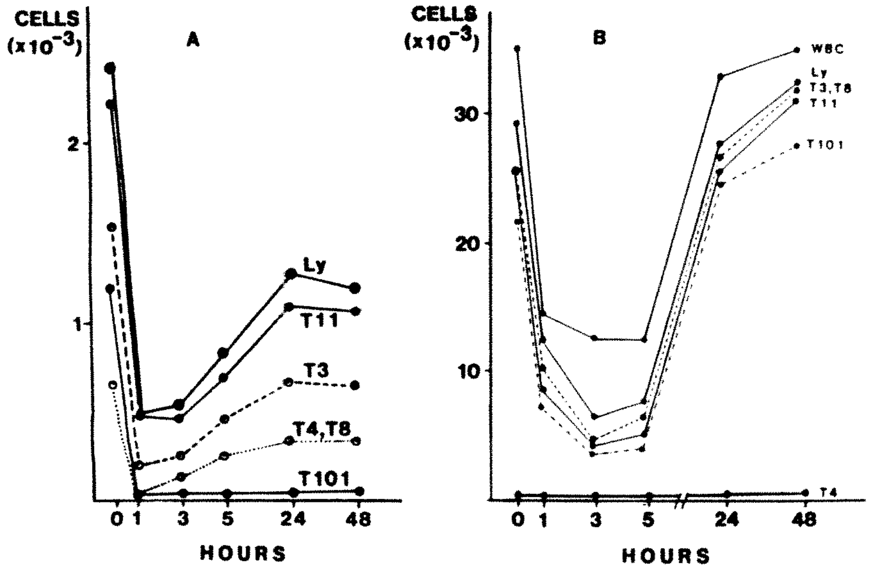

Fig 1. Effect of T101 antibody on peripheral blood lymphocytes. The two-hour antibody infusion was begun at time 0. (A) represents the observed changes in the absolute number of lymphocytes (Ly) and the T cell subsets T3, T4, T8, T11, and T101 in patient 1 (convoluted T cell lymphoma) after administration of a 50-mg dose. (B) provides this information for patient 12 (T cell CLL) following a 25-mg dose.

CTCL had a small percentage of T65-positive cells (ranging from 18% to 87%). In the leukemic patients, 65% or more of the lymphoblasts carried the T65 phenotype (Table 1). Identical results were obtained when whole blood instead of isolated, washed lymphocytes were used. This finding indicates that blocking materials such as tumor antigen or antimouse antibodies were absent prior to T101 therapy and was also confirmed in independent assays.

Kinetics and efficacy of MoAb T101–mediated peripheral blood lymphocyte removal. The kinetics of T cell targeting and removal were very similar in all nonleukemic patients (peripheral white blood count less than 10,000/μL). A steep decline in peripheral blood T cells occurred approximately one to three hours after beginning the antibody infusion, and the nadir was reached shortly thereafter (Fig 1A). Lymphocyte depletion, however, was only transient, and pretreatment levels were reached within 24 to 72 hours. The T3-, T4-, T8-, and T11-positive lymphocyte subsets were equally well removed. This finding may not be surprising since the T65 antigen is present on almost all mature T cells. The 1-mg dose caused a 63% mean reduction in peripheral blood lymphocyte counts (Table 3). Higher doses occasionally removed as much as a 96% of the T65-positive cells. The observed degree of reduction, however, was highly variable from patient to patient and within an individual patient. Dose escalation overall resulted in a steady decline in the mean reduction of peripheral blood lymphocytes, and the 10-mg MoAb dose provided the best mean peripheral blood T cell reduction.

Table 3. Reduction of Peripheral Blood Lymphocytes/Lymphoblasts After Various Doses of T101

Dose of T101 (mg)	Range of Reduction (%)	No. Treated	Mean Reduction
Nonleukemic patients			
1	51-73	3	63
10	61-95	4	87
50	3-95	7	67
100	15-96	7	60
500	0-30	4	17
Leukemic patients			
10	24	1	24
25	78-85	2	82
35	73	1	73
50	54	1	54
100	3	1	3
500	21	1	21

The nadir of the leukocyte count in leukemic patients (peripheral white blood more than 30,000/μL) occurred three to five hours after the beginning of the antibody infusion (Fig 1B). Optimal removal was observed with 25 to 35 mg of MoAb T101, and as with the nonleukemic patients, dose escalation beyond this level did not seem to enhance T cell removal (Table 3).

Influence of antigenic modulation on in vivo targeting and removal of peripheral blood lymphocytes. Antigenic modulation, ie, the selective, antibody-induced loss of a surface antigen, was studied initially in vitro by incubating lymphocytes from two patients and two normal individuals with various doses of MoAb T101 for as long as 24 hours. Concentrations of the T65 antigen began to decline two hours after addition of the antibody, and almost all incubated cells had lost their T65 antigen at the end of the incubation period. Antigenic modulation proceeded slowly for the initial 12 hours and rapidly therafter.

The in vivo pattern of antigenic modulation is demonstrated in Fig 2 (patient 5). The initial 10-mg dose already resulted in substantial serum levels of T101 MoAb. Antigenic modulation occurred at approximately five hours. Antigenic modulation did not interfere with the initial phase of targeting and lymphocyte depletion. An almost identical pattern was seen with the second (50 mg) dose. Profound antigenic modulation developed, which again did not appreciably impair T cell binding of T101 and lymphocyte depletion. Subsequent treatments (100 mg and 500 mg) seemed ineffective. T101-positive cells were undetectable even at the beginning of therapy. Persistence of bioavailable MoAb T101 had caused sustained antigenic modulation and pre-

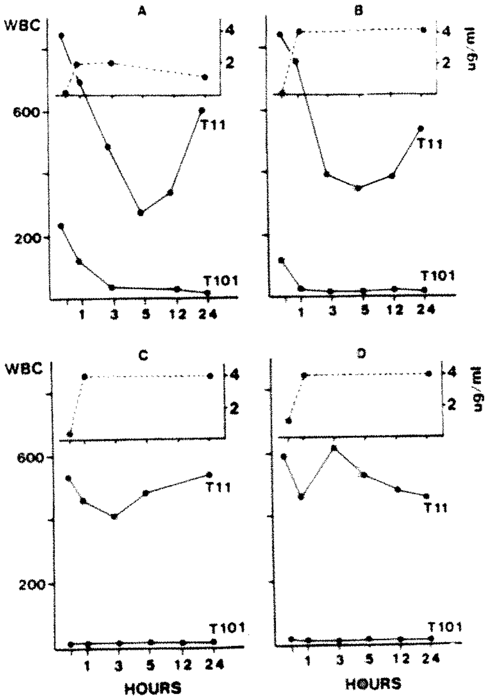

Fig 2. In vivo kinetics of antigenic modulation in patient 5. T101 doses of 10 mg (A), 50 mg (B), 100 mg (C), and 500 mg (D) were administered at time 0 by a two-hour intravenous infusion. The effect of these doses on the absolute numbers of the T11 and T101 T cell subsets is shown. Serum concentrations of T101 during each of the treatments are indicated in the inserts in each panel.

vented targeting and removal of peripheral blood lymphocytes. In this patient and also in patients 7 and 10, antigenic modulation persisted during almost the entire month of therapy and caused a progressive decline of the T101 subset (Fig 3). The rapid infusion schedule thus could not prevent sustained antigenic modulation after the repeated administration of large MoAb T101 doses.

Although peripheral blood T cells usually declined after administration of MoAb T101, a marked increase in circulating lymphocytes occurred in two patients with CTCL after several treatments. These cells were T3- and T11-positive but negative for the T65 antigen. Antigenically modulated T cells thus had entered the peripheral circulation.

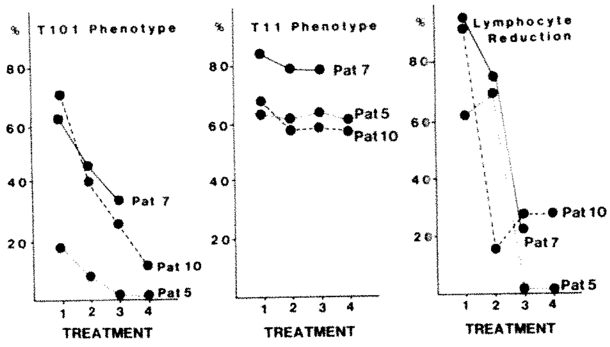

Fig 3. Influence of antigenic modulation on the expression of the T101 and T11 lymphocyte subsets and on peripheral blood lymphocyte removal. The T101 and T11 lymphocyte subsets were determined prior to each of the four treatments. In contrast, percentages of lymphocyte reductions are those obtained during therapy.

Detection of T101 MoAb in malignant tissue. Skin biopsy specimens from two patients with CTCL were evaluated by the immunoperoxidase technique for expression of the T101 and T3 antigens and for the presence of in vivo bound T101 antibody. Biopsy specimens were obtained at four hours and 24 hours after administration of 50 mg of T101. At four hours in vivo bound T101 was present in perivascular areas of the biopsy specimens. Addition of exogenous T101 MoAb revealed the T65 antigen to be abundant in the unstained areas. After 24 hours, however, neither in vivo bound T101 antibody nor the T65 antigen was detectable. The T3 antigen, however, was present in concentrations similar to those observed in the four-hour specimen. Thus, MoAb T101 had entered the target tissue by four hours, and by 24 hours its concentration was sufficient to cause antigenic modulation.

Liberation of blocking materials during T101 therapy. Although none of our patients' sera prevented in vitro targeting of peripheral blood cells with T101 prior to therapy, such blocking activity transiently developed in patient 6 and to a lesser degree in patients 1 and 12 during their initial treatment. This blocking activity significantly lowered T101 serum concentrations in patient 6 during the first and second treatment (Fig 4) and persisted for at least 48 hours (data not shown). Removal of peripheral blood lymphoblasts was impaired in the first (10 mg) treatment and was still less than optimal in the second (50 mg) treatment. In subsequent treatments no more blocking activity was detectable, and excess MoAb had induced antigenic modulation. Since assays for antimouse antibodies were negative, this blocking activity in the serum is assumed to represent liberated T65 antigen.

Determination of antimouse Ig antibodies. Antimouse antibodies were detected in three patients approximately 3 to 4 weeks after initiation of therapy. Two patients (no. 9 and 11) had CTCL, and the third patient (no. 1) had convoluted T cell lymphoma. The presence of these antibodies led to a severe anaphylactic reaction in patient 9 immediately after the start of the third antibody infusion (500 mg). Administration of this dose was delayed for 2 weeks because of noncompliance, and this interruption of therapy might have facilitated the immunization process. Interestingly, antimouse antibodies developed in patient 11 shortly after a medically necessary 2-week delay of therapy. No adverse reactions were noted clinically, but all subsequently administered MoAb doses were ineffective. In patient 1, low levels of antimouse antibodies were present at completion of the four antibody treatments. They increased to very significant levels in the 2-month follow-up period, and at the time of retreatment, antibody excess could not be achieved, and there was no therapeutic benefit.

Determination of serum concentrations of T101 antibody. Serum concentrations of immunoreactive MoAb T101 were determined in all 13 patients serially, both during and after each treatment. The highest and most consistent

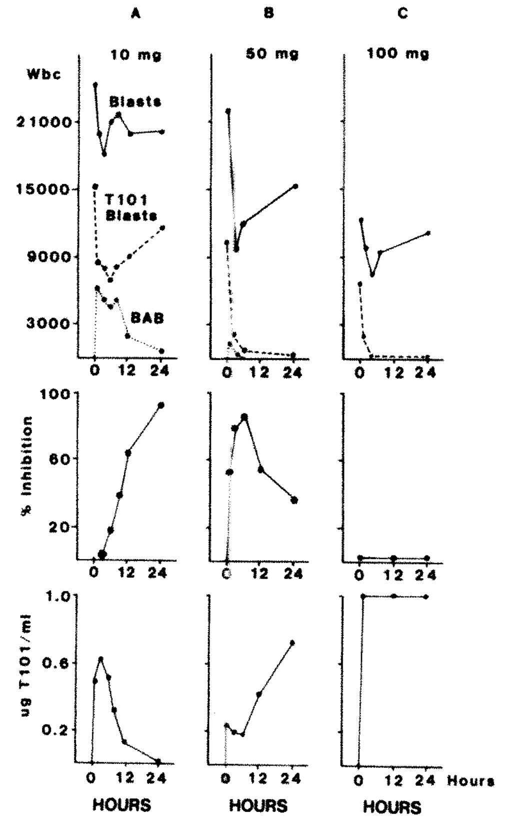

Fig 4. Influence of various T101 doses and blocking serum factors (middle panels) on removal of peripheral blood lymphoblast and T101-positive blasts (upper panels). Serum concentrations of T101 during each of the treatments are illustrated in the lower panels. T101-positive but not removed peripheral blood lymphoblasts are referred to as BAB (bound antibody).

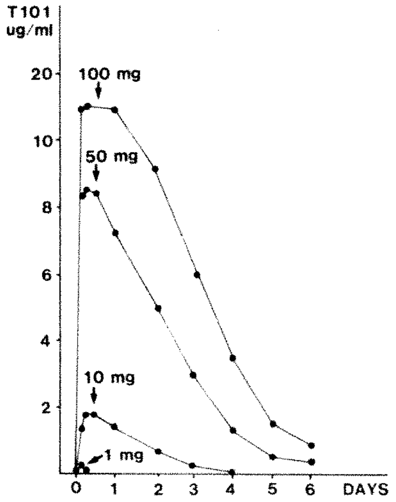

Fig 5. Serum concentrations of T101 antibody in patient 2 after administration of various antibody doses.

serum concentrations were measured in patients 2, 5, and 10, who had minimal tumor burdens and low peripheral blood counts. In these three patients, serum concentrations of the T101 antibody ranged from 0.2 μg/mL for the 1-mg dose to almost 20 μg for the 100-mg dose. T101 serum levels for patient 2 are shown in Fig 5. The 1-mg dose was only transiently detectable, whereas 10 mg of antibody was present for as long as 96 hours. Serum levels of T101 were still detectable seven days after administration of the 50- and 100-mg doses and were measured at 170 ng/mL and 750 ng/mL, respectively. In patient 5, 480 ng of T101 was present in the serum 2 weeks after administration of the final 500-mg dose. T101 serum concentrations decreased with increasing numbers of peripheral blood T cells, and a minimum of 25 to 35 mg of MoAb were required for induction of antibody excess in the leukemic patients.

The time-concentration curves of MoAb T101 were subjected to pharmacokinetic analysis. The antibody was found to follow a single compartment distribution. Serum half-life was determined to be 23, 27, and 36 hours for the 10-, 50-, and 100-mg doses, respectively.

Influence of T101 antibody on lymphoproliferation. Lymphoproliferative assays with concanavalin A or alloantigen were performed serially in all patients during and after therapy to examine the potential influence of MoAb T101 on this parameter of immunity. Lymphoproliferation was absent in the four patients with the various leukemias. Low levels were present in almost all patients with CTCL (patient 5, Fig 6). A transient decline of lymphoproliferation occurred in all responsive patients during the first three hours of antibody treatment, which corresponded with the peak of T101-induced cell removal. Proliferation usually returned to baseline at 24 hours but did not improve with the four courses of therapy. Similar low levels of lymphoproliferation were observed in patient 1 prior to each therapy (Fig 7). However, this patient's lymphocytes obtained during and after antibody infusion were highly reactive to allogeneic or mitogenic stimuli during all four treatments. Proliferative response was maximal at one hour and remained elevated during the first 24 hours after therapy. In this patient the T101 antibody itself was also strongly mitogenic to both untreated and T101-exposed mononuclear cells (Fig 7). Such responses were not obtained in any of the other patients, nor were they present in six normal controls.

Antitumor response to T101 antibody. Antitumor activity of various degrees was observed in four patients. A complete remission was obtained in patient 1 with convoluted T cell lymphoma. This patient had minimal bone marrow

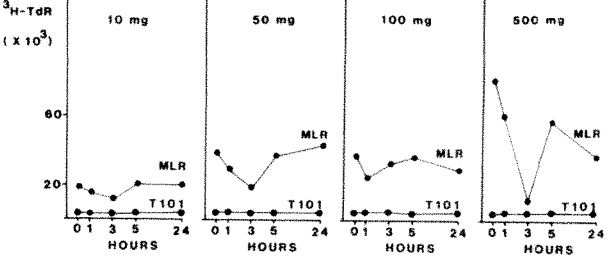

Fig 6. Effect of T101 therapy on the proliferative response of mononuclear cells from patient 5. Mononuclear cells were isolated before T101 therapy and at 1, 3, 5, and 24 hours after the beginning of the antibody infusion. Isolated cells were incubated either with alloantigen (mixed lymphocyte reaction) or with T101.

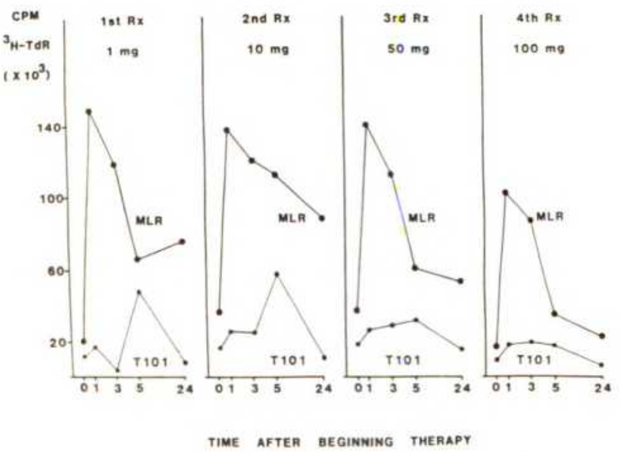

Fig 7. Effect of T101 therapy on the proliferative response of mononuclear cells from patient 1. Mononuclear cells were isolated before T101 therapy and at 1, 3, 5, and 24 hours after the beginning of the antibody infusion. Isolated cells were incubated either with alloantigen (mixed lymphocyte reaction) or with T101.

involvement as the only site of disease. After completion of therapy with T101 MoAb, bilateral bone marrow aspirates and biopsy specimens revealed a normal morphology and a blast count of 3% (Fig 8). The bone marrow biopsy was repeated shortly thereafter to minimize the risk of a sampling error, but was again negative for tumor. Remission was sustained for 8 weeks as documented by two monthly bilateral bone marrow biopsies. At relapse, retreatment with T101 antibody failed to induce an additional response. Patient 5 with CTCL sustained a partial remission. Improvement in his skin disease was noted 2 weeks after antibody therapy was begun, and more than 50% of all skin lesion had flattened at completion of therapy. Skin biopsy specimens were obtained at three previously involved sites and revealed absence of tumor. Partial remission status lasted for 3 months. Less significant antitumor responses were noted in patient 12 with transient improvement in cutaneous disease, and intermittent reduction in lymphadenopathy was found in patient 6. A reduction in circulating leukemic/Sézary cells lasting 2 weeks was observed in patients 6, 12, and 13.

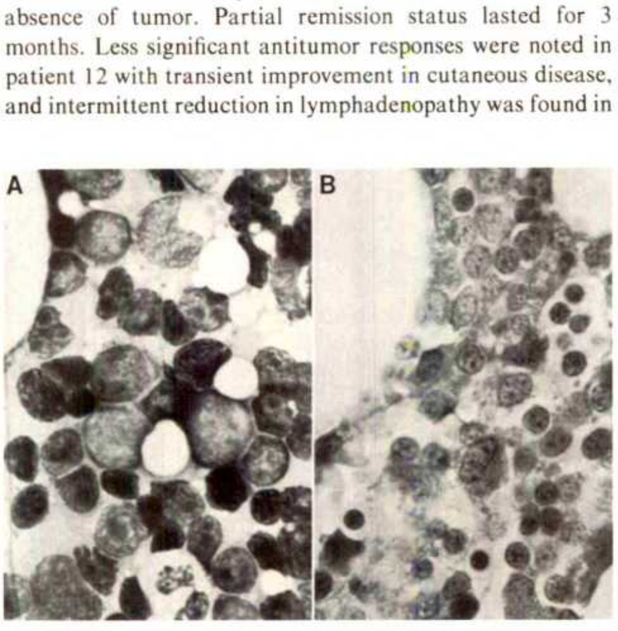

Fig 8. Representative bone marrow sections of patient 1 prior to (A) and after completion of therapy (B).

DISCUSSION

We have investigated the toxicity, pharmokinetics, and clinical efficacy of the T101 MoAb in 13 patients with various T cell malignancies. MoAb was administered as a short-term, two-hour infusion, a schedule intended to minimize the negative effects of antigenic modulation. It was based upon the in vitro observation that T101-exposed lymphocytes required at least 12 hours for conversion to complete antigen negativity. It thus was hoped that targeting and removal of peripheral blood lymphocytes occurred prior to the development of antigenic modulation.

The findings of this trial clearly support this concept. Every patient had excellent targeting and removal of peripheral blood T cells in the initial treatment regardless of the administered dose. With large MoAb doses (50 mg or above), however, bioavailable antibody persisted to the next treatment and kept peripheral blood T cells in the modulated stage. In some patients, antigenic modulation persisted during the entire month of treatment and caused a progressive decline of the T101 subset. This persistence of antigenic modulation after administration of larger doses explains why 10 mg of MoAb was the most effective dose in nonleukemic patients. Serum concentrations of MoAb T101 decreased with increasing numbers of peripheral blood T cells, and 25 to 35 mg of T101 was the equivalent most effective dose in leukemic patients. Since antigenic modulation was insignificant during the original treatment, consecutive use of higher doses would have been equally effective provided spacing of these treatments would allow for reexpression of the T65 phenotype.

Antigenic modulation was not restricted to the peripheral blood but was also present in skin biopsy specimens of patients with CTCL. Similar observations were obtained by other investigators[9,13] and confirmed the ability of MoAb T101 to enter peripheral tissues in appreciable concentrations. Besides antigenic modulation, blocking serum factors interfered with targeting and removal of peripheral blood T cells by MoAb T101. Antimouse antibodies were present in three patients of this study. This is less than reported by other investigators[8,13] and might be related to our treatment schedule, which consisted of only two-hour treatments for each patient. Desensitization to the mouse protein was recently observed after administration of very high MoAb doses[14] but seems a more remote possibility since much smaller MoAb doses were administered in this trial.

An anaphylactic reaction developed in one of these three patients after administration of the third MoAb dose. There were no such side effects, however, in the other two patients, and the pathophysiologic base for this discrepancy is presently unknown. We examined possible differences in immunoreactivity with lymphoproliferative assays but found similar responses to concanavalin A or alloantigens in all three patients. Lymphoproliferative assays were also not able to predict the development of antimouse antibodies in general, since all patients with CTCL had similar low levels of lymphocyte reactivity. The presence of antimouse antibodies usually rendered further therapy with MoAb ineffective, although some lymphocyte removal occurred in one patient in the early stage of the immunization process.

Blocking materials other than antimouse antibodies were detected in several of our patients during therapy. Blocking of T101 activity occurred early in the treatment course and caused lowered serum concentrations of administered MoAb T101. The blocking apparently was due to tumor antigens in the serum and seemed to be generated by MoAb-mediated cell destruction because antigenic modulation was not present. A similar blocking activity was detected by Miller et al in one patient after administration of a 1-mg dose[6] and was identified as the T65 antigen.

Aside from pruritus, hives, and other effects commonly associated with therapy with mouse MoAbs, previously unreported toxicities occurred during this trial. Substantial blood pressure changes, premature atrial contractions, and atrial fibrillation and atrioventricular conduction disturbances were noted. These toxicities occurred at the 10-mg dose level but were more frequent with doses of 50 mg or higher. They seemed to be associated with the rate of antibody infusion, since administration of identical MoAb doses by a continuous 24-hour infusion was much less toxic. This assumption is also supported by a trial of T3 MoAb in patients with renal graft rejection (Goldstein et al, unpublished observation) where administration of large quantities of MoAb over a short time period resulted in similar toxicities. MoAb T101 is thus only poorly suited for rapid administration. In the proper clinical setting, however, doses of up to 50 mg can be delivered without life-threatening toxicity.

A poorly understood side effect was moderate and occasionally severe shortness of breath, which occurred in almost all patients of this trial. A similar pulmonary toxicity was noted in the T101 trials of Dillman et al[9] and Foon et al.[10] In two of these patients, transient defects were noted either on a perfusion lung scan or chest x-ray.[15] MoAb T101 thus might have formed lymphocyte aggregates that served as microemboli. Prolongation of the antibody infusion from two hours to 24 hours[8] or 50 hours[10] abolished this side effect, and the rapid rate of infusion seemed the cause for this toxicity. Antigenic modulation that invariably occurred with prolongation of the infusion might be an alternative explanation. In our trial, shortness of breath was most pronounced during each patient's initial treatment, and subsequent use of immunomodulating doses either diminished or altogether abolished this side effect.

Various degrees of antitumor responses were observed in this trial. They ranged from transient reductions in peripheral T cell counts to sustained remissions in two patients, with disease restricted to bone marrow (patient 1) or skin (patient 5). The best antitumor responses thus were not obtained in leukemic patients but in those with tissue-based disease (bone marrow, skin). This seems surprising since these patients' malignant T cells were not directly available to the filtering action of the reticuloendothelial system (RES). Effector systems other than the RES thus might have mediated the antitumor activity, and the marked improvement in the mixed lymphocyte reaction in patient 1 supports this notion. We, however, also noted an influx of antigenically modulated T cells into the circulation in two patients with CTCL. This included patient 5 and occurred after each treatment. Dillman et al found a similar phenomenon in one patient with CTCL,[9] and it thus appears that tissue-based T cells were able to enter the peripheral blood stream. These cells might have undergone subsequent removal by the RES.

In summary, this trial provided an exciting opportunity to analyze the action of MoAb T101 at a molecular level. Therapy was specific but only modestly efficacious. Rapid infusion of doses that did not cause antigenic modulation provided excellent targeting and removal of peripheral blood T cells and might be a valid approach in future trials, particularly with immunoconjugated T101.

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Expression of Antigens of the Platelet Glycoprotein IIb/IIIa Complex on Human Hematopoietic Stem Cells

By John K. Fraser, Michael F. Leahy, and Michael V. Berridge

Several cell surface and cytoplasmic markers specific for the megakaryocyte-platelet lineage have been described. However, as yet, none of these has been shown to be expressed on cells earlier than the committed megakaryocyte progenitor, CFU-Meg. The present study was aimed at determining whether platelet lineage antigens could be detected on human pluripotential stem cells. Rabbit antiserum against human platelets (APS) was extensively absorbed with erythrocytes and either platelets, neutrophils, monocytes, or cells of the monocytic cell line U937. The anti-stem cell antibodies in each absorbed antiserum were determined using a complement-dependent cytotoxic assay for the pluripotential stem cell CFU-mix. Platelets alone removed anti-stem cell antibodies from APS. Absorption of APS with platelets from a patient with Glanzmann's thrombasthenia failed to remove the anti-stem cell activity, providing evidence for involvement of the platelet glycoprotein IIb/IIIa complex. Antiserum against purified glycoprotein IIb and against glycoprotein IIIa also recognized stem cells, resulting in reduced formation of mixed colonies. Absorption of these antisera with normal platelets removed the anti-stem cell activity, indicating that both IIb and IIIa are represented on stem cells. Hence, cell surface antigens specific for the stem cell-megakaryocyte-platelet pathway are expressed on the platelet glycoprotein IIb/IIIa complex.

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THE QUEST for markers specific for the megakaryocyte-platelet lineage has been the subject of much research in recent years. In rodents the presence of acetylcholinesterase has proved to be a useful marker,[1,2] and more recently platelet peroxidase has been used in the human system.[3] Cell surface markers have also been used to define platelet-forming cells. Thus, Mazur et al have used rabbit antiserum against a human platelet glycoprotein extract to fluorescently label bone marrow and cell culture-derived megakaryocytes.[4] This antiserum immunoprecipitated three proteins of molecular weight (mol wt) 145,000, 125,000, and 95,000. More recent studies have shown that antigenic determinants of the platelet glycoprotein IIb/IIIa complex, as recognized by the monoclonal antibodies J15 (IIb/IIIa) and C17 (IIIa only), are coexpressed with platelet peroxidase on the earliest identifiable megakaryocyte percursor—the promegakaryoblast.[5] Such cells are also recognized in vitro among cells derived from CFU-Meg as early as day 5 of culture.[6] Furthermore, Levene et al[7] have demonstrated that monoclonal antibodies capable of immunoprecipitating IIb/IIIa also recognize the committed progenitor CFU-Meg itself.

The demonstration that fibrinogen and fibrin fragment D, both of which bind to platelets through the glycoprotein IIb/IIIa complex, stimulate stem cell growth[8] suggests that these molecules may directly bind to stem cells that might therefore express a fibrinogen/fibrin D receptor. In this context, the tumor cell line K562, which appears to share many features in common with early hematopoietic cells, has been shown to express antigenic determinants of the platelet glycoprotein IIb/IIIa complex.[9] In other studies it has been demonstrated that induction of differentiation of K562 cells with sodium butyrate results in a small degree of megakaryoblast production.[10] These studies were extended by Tetteroo et al[11] who showed that 12-0-tetradecanoylphorbol-13-acetate (TPA) induction of K562 results in an increase in C17 (glycoprotein IIIa-specific) positivity from 1% to 18%. Together, this evidence suggests that antigens of the platelet glycoprotein IIb/IIIa complex may be expressed on the hematopoietic stem cell and that such antigens might be useful as markers of the stem cell-megakaryocyte-platelet lineage.

In the present study, rabbit antisera against human platelets (APS), glycoprotein IIb (AIIbS), and glycoprotein IIIa (AIIIaS) have been prepared and extensively absorbed with monocytes or with cells of the monocytic cell line U937 to produce platelet-specific antisera. These antisera have been used in a complement-dependent cytotoxic assay against CFU-mix to provide evidence that the platelet glycoprotein IIb/IIIa complex expresses stem cell-megakaryocyte-platelet lineage-specific antigens. In support of these results, platelets from a patient with Glanzmann's thrombasthenia, which are known to exhibit greatly reduced IIb/IIIa expression, were unable to absorb the anti-stem cell activity in APS whereas normal platelets efficiently removed this activity.

MATERIALS AND METHODS

Preparation of APS. Platelets for immunization and absorption were purified from platelet transfusion packs by repeated differential centrifugation. Platelet suspensions were centrifuged at 200 g for ten minutes and aspirated platelets harvested at 1,600 g for eight minutes. Contamination with erythrocytes and leukocytes was less than 1 cell per 5,000 platelets.

APS was prepared by immunizing a New Zealand white rabbit at multiple subcutaneous sites with 2×10^9 platelets homogenized in complete Freund's adjuvant. Five weeks later the animal was given three intravenous booster injections of 1×10^9 platelets at weekly intervals. Four days after the final boost, the rabbit was bled from the ear and the serum collected, heat inactivated at 56 °C for 30 minutes, and stored at −20 °C.

Peripheral blood cell purification. AB Rh+ erythrocytes were purified from whole blood collected into citrate-phosphate-dextrose (CPD) by repeated washing and centrifugation at 1,600 g for ten

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minutes with repeated removal of the buffy coat. WBC contamination was less than 0.2%.

Lymphocytes and neutrophils were prepared from buffy coats by density centrifugation over a two-step gradient of Ficoll-Hypaque (Nyegaard, Oslo) (ρ = 1.077 and 1.09 g/mL) at 400 g for 30 minutes. Neutrophils harvested from the interface of these two solutions were 95% pure with 5% lymphocyte contamination. Lymphocytes were harvested from the blood–Ficoll-Hypaque interface, platelets being removed by repeated centrifugation at 200 g for five minutes. The final lymphocyte purity was 80% to 90% with 10% to 20% monocyte contamination.

Monocytes were purified from the buffy coats of CPD-blood using hypertonic saline conditioning as described by Recalde.[12] The monocyte purity was 75% to 85% with 15% to 25% lymphocyte contamination and low variable platelet contamination because of platelet-monocyte adherence.

Preparation of human bone marrow cells. Human bone marrow was drawn from normal donors after informed consent and in accordance with institutional guidelines using heparin as an anticoagulant. Mononuclear cells were isolated from the interface with Ficoll-Hypaque (ρ = 1.077) after centrifugation at 400 g for 30 minutes and washed twice.

Absorption of antisera. Absorption of antisera was carried out by consecutive incubations with an equal volume of purified packed cells at 4 °C for 40 minutes. All APS samples used in this study were basally absorbed with AB Rh+ erythrocytes (four absorptions). Additional absorptions are referred to in the text and consisted of two consecutive incubations with an equal volume of packed cells. Heat-inactivated, absorbed antisera were used directly in immunofluorescence, radioimmunoassay, immunoprecipitation, platelet aggregation, and cytotoxicity studies.

Immunofluorescence. Cells for fluorescence (2×10^5) were washed two to three times in phosphate-buffered saline (PBS), resuspended in 25 μL of primary antibody at 4 °C for 40 minutes, then washed, and incubated at 4 °C for 40 minutes with 25 μL of 1:16 dilution of fluorescein isothiocyanate (FITC) goat antirabbit Ig (Behring, Marburg, West Germany). Fluorescence was determined in a Zeiss Photomicroscope II with epifluorescence.

Radioimmunoassay. Cells were fixed to flexible polyvinyl chloride plates (Nunclon, Roskilde, Denmark) using the method of Stocker and Heusser.[13] Briefly, cells in PBS (100 μL of 6×10^5 cells/mL) were pipetted into each well and the plate centrifuged at 150 g for one minute. Glutaraldehyde (50 μL, 0.25% in PBS) was then gently added to each well and the plates again centrifuged briefly. Five minutes later the glutaraldehyde was removed and the plates washed several times in PBS containing 1% bovine serum albumin (BSA) and 0.05% sodium azide. Wells were inspected using an inverted microscope to ensure uniform fixation and the plates stored in PBS containing 0.1% BSA at −20 °C. Affinity-purified IgG goat antirabbit Ig (a gift from Dr M. Bodger, Christchurch Hospital) was iodinated using chloramine-T following the procedure outlined by Chard.[14] The labeled antibody had a specific activity of 4.2×10^7 cpm/μg protein. For the assay, 50 μL of antiserum was added to each well. After incubation at 20 °C for one hour the wells were washed three times with PBS containing 1% BSA. ^{125}I-labeled goat antirabbit Ig (50 μL, 3×10^4 cpm) was added to each well and the plates incubated for one hour at 20 °C. Plates were washed five times in PBS containing 1% BSA and individual wells excised using a hot wire and counted in a gamma counter.

Cell lines. U937 was obtained from Dr J. McKenzie, Christchurch Clinical School; KG-1a from Dr M. Bodger, Cytogenetics Unit, Christchurch Hospital by kind permission of Dr D.W. Golde; HEL from Dr T. Papayannopoulou, Seattle; and K562 from Dr G. Findlay, University of Auckland School of Medicine. These cell lines were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with glutamine, antibiotics, and 10% fetal calf serum. The 5637-conditioned medium was prepared by seeding 5×10^5 5637 cells obtained from Dr M. Bodger, Christchurch Hospital, into 25 mL Iscove's modified Dulbecco's medium (IMDM, GIBCO) supplemented with antibiotics and 10% fetal calf serum. The cells were allowed to expand for eight to ten days in 80-cm^2 culture flasks (Nunclon) in an atmosphere of 5% CO_2 in air, after which the supernatant was centrifuged, filtered through an 0.45-μm filter, and stored frozen at −20 °C.

Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Platelet surface proteins were labeled with ^{125}I by a modification of the lactoperoxidase method of Hubbard and Cohn.[15] Between 40- to 60-μL packed cell volumes of washed platelets were incubated for 20 minutes at 20 °C in the presence of 100 μL of 1 mg/mL lactoperoxidase (Sigma Chemical Co. St Louis), 0.5 mCi carrier-free sodium ^{125}I-iodide (Amersham, Sydney, Australia), 20 μL of 1 mg/mL glucose oxidase (Sigma), and 20 μL of 50 mmol/L glucose. The reaction was stopped by the addition of 1 mL of ice-cold 5 mmol/L KI in PBS and the cells lysed in solubilization buffer (50 mmol/L Tris-HCl and 1% sodium deoxycholate, pH 7.4). The lysate was absorbed three times with 20 μL of packed glutaraldehyde-fixed *Staphylococcus aureus*. Absorbed lysate (50 μL) was then incubated with 10 μL antiserum at 4 °C for 40 minutes, after which 30 μL of packed *S aureus* was added and incubated for 40 minutes. Antibody-coated *S aureus* was washed three times with solubilization buffer and then three times with 50 mmol/L Tris-HCl, pH 7.4. Immunoprecipitated proteins were solubilized by incubation at 100 °C for 15 minutes in 100 μL 1:3 SDS-sample buffer (0.15 Tris-HCl, pH 6.8; 3% SDS; 15% glycerol; and 0.003% bromophenol blue) in the presence or absence of 10% 2-mercaptoethanol. The insoluble *S aureus* was removed by centrifugation, and 90 μL of the sample was loaded onto a slab polyacrylamide gel with a 4.5% stacking layer and a 5% to 15% resolving gel. The gel was run at 20 mA through the stacking layer and 50 mA through the resolving layer. The gel was stained with Coomassie blue, dried, and autoradiographed using Kodak R-X-OMat (Rochester, NY) film at −70 °C.

Purification of platelet glycoproteins IIb and IIIa by Sepharose 4B–concanavalin A affinity chromatography. Concanavalin A (Con A) (Calbiochem, San Diego) was coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) at a concentration of 5 mg/mL beads in the presence of 2% α-methyl mannoside (Sigma) to protect the binding site. The Con A affinity column (5 mL) was equilibrated with solubilization buffer and then mixed end over end for two hours at room temperature with 10 mL solubilized platelets (equivalent to 2×10^{10} platelets). The column material was washed several times with solubilization buffer and eluted by two hours of end-over-end mixing with 5 mL of solubilization buffer containing 2% α-methyl mannoside.

Eluted proteins were run on SDS-PAGE as previously described and the bands corresponding to glycoproteins IIb and IIIa (Fig 1) cut out and used to immunize two rabbits using the method of Boulard and Lecroisey.[16]

Platelet aggregation. Antibody-mediated inhibition of platelet aggregation was determined by incubating 400 μL of platelet-rich plasma obtained from blood collected into citrate (platelet count, 4 to 5×10^8/mL) with 50 μL of antibody at 20 °C for 20 minutes. Aggregation was initiated by addition of 50 μL of adenosine diphosphate (ADP) cluster reagent (Dade, Miami) and aggregation followed using a Corning EEL 169 platelet aggregometer. Inhibition of platelet aggregation was measured at plateau light transmission, 100% transmission (aggregation) being defined as the difference between platelet-poor plasma and platelet-rich plasma.

Progenitor and stem cell cytotoxicity. Mixed colonies were grown using a modification of the method described by Fauser and

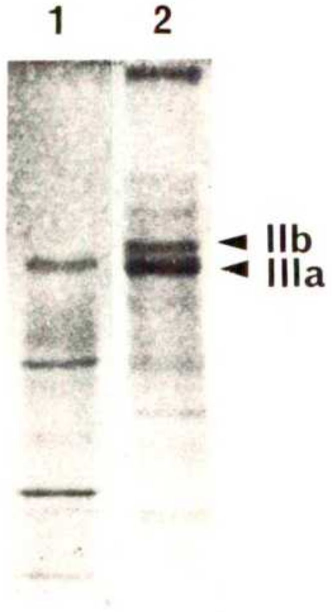

Fig 1. **Purification of platelet glycoproteins IIb and IIIa. Solubilized platelet glycoproteins were separated by Sepharose 4B–Con A affinity chromatography and the eluate analyzed by SDS-PAGE under nonreducing conditions and stained with Coomassie blue. (Lane 1) Molecular weight markers: phosphorylase b, 97,000; albumin, 67,000; carbonic anhydrase, 30,000; and soybean trypsin inhibitor, 20,100. (Lane 2) Affinity eluate of the Sepharose 4B–Con A column showing purification of glycoproteins IIb and IIIa.**

Messner et al.[17] Cord blood was collected into preservative-free heparin (40 U/mL blood) or sterile CPD and mononuclear cells separated by density centrifugation at 400 g for 30 minutes over Ficoll-Hypaque (ρ = 1.077 g/mL). Mononuclear cells (2×10^6) were incubated at 4 °C for 40 minutes with 25 μL of diluted antiserum, after which 25 μL of rabbit complement obtained from 17-day-old rabbits and diluted to 1:3 in IMDM was added and incubation continued at 37 °C for 60 minutes. Prior to use the 17-day rabbit complement was absorbed twice at 4 °C for 30 minutes with an equal volume of cord blood buffy coat cells to remove nonspecific activity. Cells were then cultured in 0.9% methyl cellulose (Fluka, Buchs, W Germany) in IMDM, 20% human plasma (either autologous cord plasma or prescreened normal AB Rh+ plasma), 10% fetal calf serum, 5% 5637 cell–conditioned medium[18] 5×10^{-5}mol/L 2-mercaptoethanol, and one unit of human erythropoietin (Toyobo, Osaka, Japan). Cells were plated out in 1-mL aliquots containing 7 to 8×10^4 cells/plate.

After incubation for 16 days at 37 °C, candidate mixed colonies were picked out with a 5-μL micropipette and resuspended in 100 mL RPMI 1640 medium containing 40% horse serum. Cells were centrifuged in a Shandon II cytocentrifuge (Shandon-Elliott Cytospin, Sewickley, Pa) and the composition of the colonies determined by May-Grünwald-Giemsa staining.

RESULTS

Table 1. Binding of APS to Mononuclear Cells and Platelets

	Fluorescence Intensity†		
Absorption of APS*	Platelets	Monocytes	Lymphocytes
None	+ + +	+ +	+
Monocytes	+ +	–	–
U937 cells	+ + +	–	–
Glanzmann's platelets	+ +	+/–	–
Normal platelets	–	–	–

*APS that had been basally absorbed with erythrocytes were additionally absorbed with 2 vol of either monocytes, U937 tumor cells, platelets from a patient with Glanzmann's thrombasthenia, or normal platelets.

†Fluorescence intensity was arbitrarily rated from weakly positive (+/–) to strongly positive (+ + +) against control rabbit serum.

Specificity of APS for normal hematopoietic cells. Rabbit antiserum against human platelets, when extensively absorbed with erythrocytes, showed high binding to platelets, intermediate binding to monocytes, and low binding to peripheral blood lymphocytes as determined by indirect immunofluorescence (Table 1). No significant binding to neutrophils was observed. Further absorptions of APS with monocytes or with U937 monocytic leukemia cells removed the activity against lymphocytes and monocytes, resulting in antiserum specific for platelets. Absorption of APS with normal platelets completely removed antibodies that bound to platelets, lymphocytes, and monocytes, whereas absorption with platelets from a patient with Glanzmann's thrombasthenia removed the lymphocyte activity, significantly reduced monocyte binding, but only slightly reduced platelet fluorescence. The binding of APS that had been absorbed with erythrocytes and U937 cells to bone marrow cells is shown in Fig 2. Less than 1% of cells showed fluorescence. Positive cells included platelets, megakaryocytes, and a population of small lymphocytelike cells that were not positively identified by phase-contrast microscopy.

Platelet lineage specificity of APS demonstrated with multipotential tumor cell lines. The binding of APS that had been absorbed with erythrocytes and U937 cells to morphologically identifiable cells of the megakaryocyte-platelet lineage and to a small population of small lymphocytelike cells in the bone marrow raised the possibility that APS may also recognize more primitive cells in this pathway. To test for the presence of platelet lineage–specific antibodies against uncommitted progenitor cells, binding of APS to the multipotential tumor cell lines K562, HEL, and KG-1a was determined and the specificity of bound antibodies examined by absorption analysis (Table 2). APS bound strongly to each of the tumor cell lines, and platelets alone absorbed all of this activity. Although peripheral blood lymphocytes and neutrophils failed to absorb tumor cell–binding antibodies, both monocytes and U937 cells were partially absorbing, results that closely parallel the platelet-binding specificity of APS shown in Table 1.

Platelet lineage specificity of APS demonstrated with cord blood stem cells (CFU-mix). Treatment of cord blood mononuclear cells with APS that had been basally absorbed with erythrocytes followed by rabbit complement resulted in complete inhibition of CFU-mix growth (Table 3). Absorption with platelets completely removed cytotoxic antibodies, whereas absorption with either lymphocytes, neutrophils, monocytes, or U937 cells had no effect on CFU-mix cytotoxicity.

Lineage specificity at the level of stem cells and progenitor cells is also shown in Fig 3. Neither APS that had been absorbed by erythrocytes nor APS that had been additionally

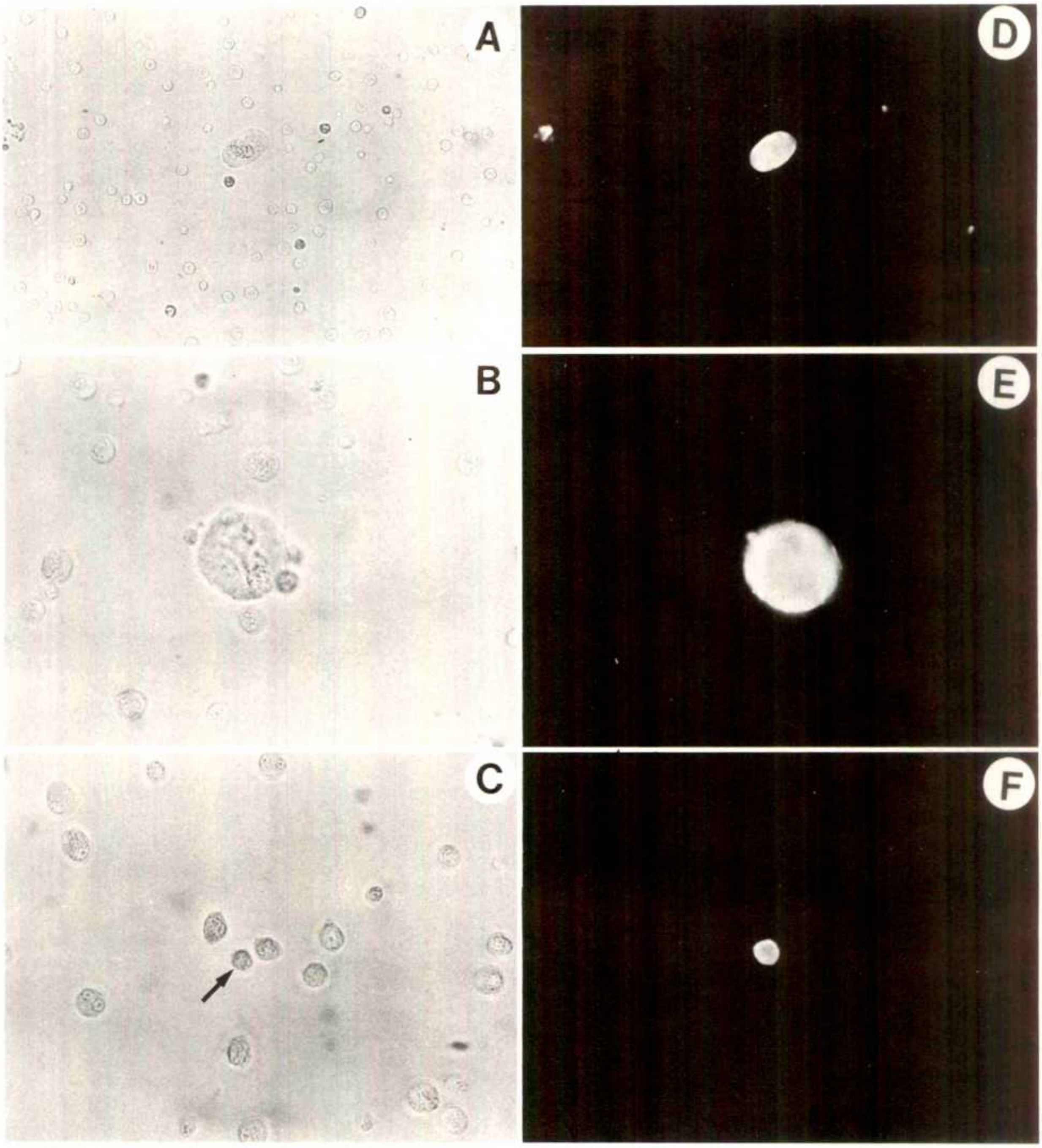

Fig 2. **Immunofluorescence of bone marrow cells stained with APS. Bone marrow cells were stained with APS that had been absorbed with erythrocytes and U937 cells followed by fluorescein-conjugated goat antirabbit IgG. (A) Bright field. (B, C) Phase contrast. (D to F) Fluorescence staining: (D) large megakaryocyte (original magnification × 200); (E) intermediate megakaryocyte (original magnification × 500; current magnification × 375); (F) small lymphocytelike cell (original magnification × 500).**

absorbed with various cell types showed any significant cytotoxic effect against the erythroid progenitor cell (BFU-E) or the granulocyte/macrophage progenitor cell (CFU-GM), results that contrast strongly with the cytotoxic effect of APS for CFU-mix where only platelet absorption reduced cytotoxicity. We have been unable to grow megakaryocyte progenitor cell colonies (CFU-Meg) from cord blood to determine APS cytotoxicity for this cell type.

Molecular identification of platelet lineage antigens recognized by APS. The results presented in Table 1 show that platelets from a patient with Glanzmann's thrombasthenia failed to remove most of the platelet-binding antibodies in APS, indicating that most of these antibodies could be accounted for by cell surface differences between normal and Glanzmann's platelets. To show that this effect also applied to those lineage-specific antibodies that bound to CFU-mix,

Table 2. Binding of APS to K562, HEL, and KG-1a Leukemic Cells

Absorption of APS*	Fluorescence Intensity† K562	Radioimmunoassay‡ (Percent Maximum Radioactivity) K562	HEL	KG-1a
None	+ + +	100	100	100
Lymphocytes	+ + +	92	94	87
Neutrophils	+ + +	98	96	97
Monocytes	+ +	61	77	48
U937 cells	+ +	69	80	72
Platelets	−	2	2	−2

*APS that had been basally absorbed with erythrocytes were further absorbed twice with either lymphocytes, neutrophils, monocytes, U937 cells, or platelets.

†Fluorescence intensity was arbitrarily rated between strongly positive (+ + +) and negative (−) against control rabbit serum.

‡Background radioactivity determined with control rabbit serum was subtracted from all results.

the ability of Glanzmann's platelets to absorb antibodies cytotoxic for CFU-mix was compared with the absorbing capacity of normal platelets (Table 3, see also Fig 3). Glanzmann's platelets were only 10% as effective at removing antibodies cytotoxic for CFU-mix as normal platelets.

Immunoprecipitation of ^{125}I-labeled platelets with APS. To further characterize the molecular nature of lineage-specific antigens recognized by APS, APS was absorbed with erythrocytes and U937 cells and used to immunoprecipitate ^{125}I surface-labeled platelets. Figure 4 shows that three major bands of mol wt 178,000, 142,000, and 99,000 were immunoprecipitated under nonreducing conditions, the latter two bands being more strongly represented (Fig 4A, lane 2). These bands correspond to platelet glycoproteins IIb and IIIa, respectively. Following reduction, six bands were observed, the major one corresponding to a merged IIb/IIIa band (mol wt 125,000 and 116,000) (Fig 4A, lane 3). This pattern of mol wt change is characteristic of platelet glycoproteins IIb and IIIa.[19]

Immunoprecipitation of ^{125}I-labeled platelets with APS that had been absorbed with platelets from a patient with Glanzmann's thrombasthenia revealed only two proteins under nonreducing conditions corresponding to glycoproteins IIb and IIIa (Fig 4B, lane 2). These proteins are compared with proteins from whole platelet lysates under reducing (Fig 4B, lane 1) and nonreducing (Fig 4B, lane 3) conditions. Thus APS absorbed with Glanzmann's platelets appears to be monospecific for the platelet glycoprotein IIb/IIIa complex.

Table 3. Effect of Cell Absorption on the Cytotoxicity of APS for CFU-Mix

Absorbing Cell Type	CFU-mix Plating Efficiency (CFU-mix/2 × 10⁵ Cells)*
None	0 ± 0
Lymphocytes	0 ± 0
Neutrophils	0 ± 0
Monocytes	0 ± 0
U937 cells	0 ± 0
Glanzmann's platelets	0.26 ± 0.26
Normal platelets	2.60 ± 0.52

*Cord blood cells were treated with antisera and absorbed rabbit complement prior to plating. Control cells treated with control rabbit serum produced 2.6 ± 0.78 CFU-mix per 2 × 10⁵ cells plated.

Properties of antisera against platelet glycoproteins IIb and IIIa. Platelet glycoproteins IIb and IIIa were prepared from eluates of Con A–Sepharose 4B by SDS-PAGE (Fig 1). Antiserum against IIIa (AIIIaS) showed strong immunofluorescence with platelets and immunoprecipitated only IIIa from radioiodinated platelets, whereas antisera against IIb (AIIbS) exhibited weak fluorescence for platelets and no immunoprecipitation products were detectable. Treatment of cord blood mononuclear cells with AIIIaS and complement completely removed cells capable of forming mixed colonies, whereas AIIbS gave partial (57%) inhibition of CFU-mix.

Effect of APS on platelet aggregation. APS that had been absorbed with erythrocytes and U937 cells strongly inhibited ADP-induced platelet aggregation (Fig 5). A prozone was observed at high antibody concentration, possibly

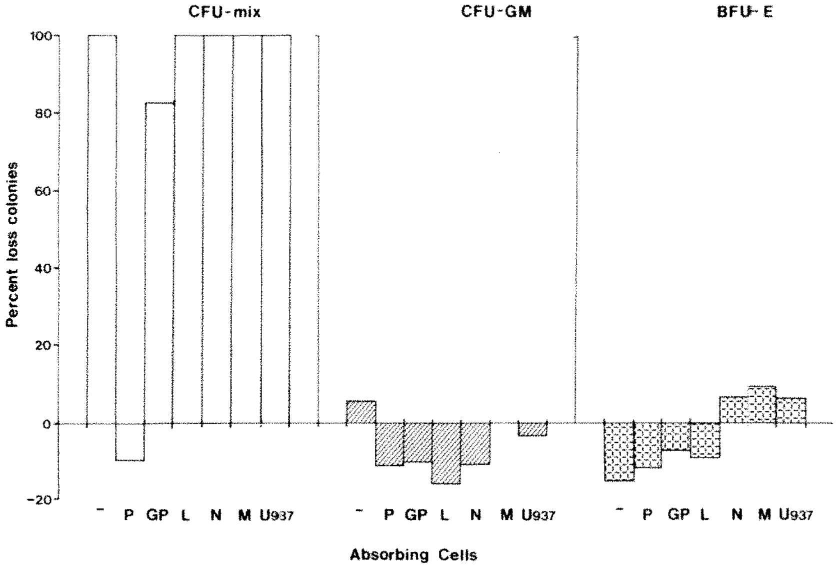

Fig 3. Cytotoxicity of APS for in vitro colony-forming cells in cord blood. Cord blood mononuclear cells (2 × 10⁶) were treated with APS that had been basally absorbed with erythrocytes and further absorbed with normal blood lymphocytes, neutrophils, monocytes, U937 tumor cells, platelets, or platelets from a patient with Glanzmann's thrombasthenia followed by rabbit complement. Cells were plated in methyl cellulose (7 to 8 × 10⁴ cells per plate) and colonies scored after 16 days, at which time control plates contained 193 ± 28 CFU-GM, 108 ± 16 BFU-E, and 3 ± 1 CFU-mix. Results are the average of three to four experiments, each experiment having triplicate plates.

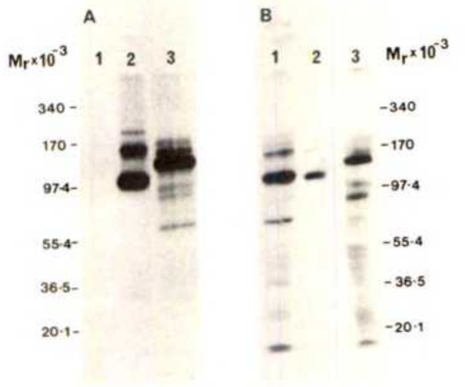

Fig 4. Immunoprecipitation of ^{125}I-labeled platelets with APS. Radioiodinated platelets were solubilized and immunoprecipitated with the following: (A) lane 1, control serum; lane 2, APS absorbed with U937 cells (nonreduced); lane 3, APS absorbed with U937 cells (reduced); and (B) lane 2, APS absorbed with Glanzmann's platelets (nonreduced). For comparison solubilized ^{125}I-labeled platelets were also analyzed directly under nonreducing (B, lane 1) and reducing (B, lane 3) conditions.

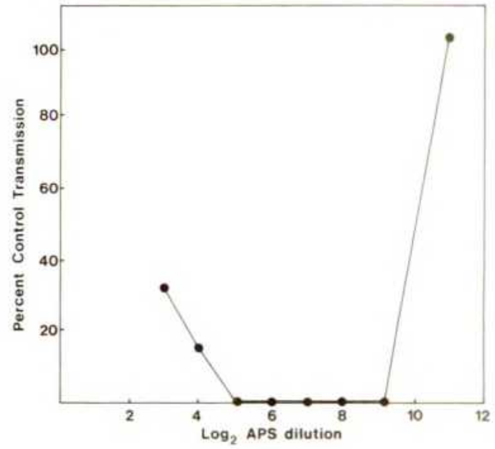

Fig 5. Inhibition of ADP-induced platelet aggregation with APS. Platelet-rich plasma was incubated with control serum or serially diluted APS at 20 °C for 20 minutes prior to inducing aggregation with ADP cluster reagent. Inhibition of transmission with antibody was measured relative to controls. One hundred percent transmission was defined as the difference in plateau transmission between platelet-rich plasma and platelet-poor plasma.

because of agglutinating antibodies in APS. Inhibition of platelet aggregation titrated out at 1:1,024 dilution of antiserum. In contrast, neither AIIIaS nor AIIbS inhibited aggregation, even at the highest final concentration of antibody attainable in this system (1:8).

DISCUSSION

In these studies we have presented evidence that the pluripotential hematopoietic stem cell expresses platelet lineage–specific markers that can be attributed, at least in part, to epitopes on the platelet glycoprotein IIb/IIIa complex. This constitutes the first description of IIb/IIIa glycoprotein expression on cells earlier than the committed CFU-Meg and supports the suggestion of Levene et al[7] that epitopes of the IIb/IIIa complex may be considered lineage-specific markers of the entire platelet-megakaryocyte lineage.

Lineage-specific antibodies in APS have been demonstrated in several different systems. First, absorption of APS with monocytes or with the monocytic cell line U937 generated an antiserum that showed specificity for platelets within the peripheral blood system. This antiserum bound to a small fraction of marrow cells (<1%) comprising megakaryocytes and a population of lymphocytelike cells (Fig 2). The platelet lineage specificity of antibodies in APS was further demonstrated in binding assays to the multipotential myeloid leukemic cell lines K562, HEL, and KG-1a where only platelets completely absorbed antibodies that bound to these cell lines (Table 2). Monocytes and U937 cells partially removed antibodies in APS that bound to these three tumor cell types, but neutrophils and lymphocytes were not absorbing. These results are consistent with studies indicating IIb/IIIa on monocytes and U937 cells,[20–22] although contrary evidence has also been presented.[23,24]

Immunoprecipitation experiments (results not shown) showed platelet glycoproteins IIb and IIIa to be the major cell surface molecules on HEL and KG-1a recognized by APS. Although we were unable to immunoprecipitate IIb and IIIa from K562 cells with APS, others have shown low levels of IIIa expression.[9] Finally, the complete lineage specificity of antibodies in APS was demonstrated with reference to the multipotential stem cell CFU-mix. Thus, CFU-mix but not CFU-GM or BFU-E were sensitive to cytotoxic antibodies in APS. Although we have been unable to grow CFU-Meg from cord blood or human marrow to test its sensitivity to APS, Levene et al[7] have previously demonstrated the expression of epitopes of the IIb/IIIa complex on CFU-Meg using complement-mediated cytotoxicity with monoclonal antibodies. Thus antibodies in APS recognize antigens expressed throughout the entire stem cell-megakaryocyte-platelet pathway.

Biochemical characterization of the cell surface molecules recognized by platelet cell lineage–binding antibodies was provided by absorbing APS with platelets from a patient with Glanzmann's thrombasthenia, a condition characterized by extensive loss of IIb/IIIa expression (<5% of normal).[25,26] The failure of these cells to remove the anti–stem cell activity from APS is in direct contrast to the results obtained with normal platelets, which completely removed the activity. These results provide evidence that the cell surface molecules deficient in the Glanzmann's condition are those that bear platelet lineage–specific antigenic markers. Immunoprecipitation of glycoproteins IIb and IIIa from ^{125}I-labeled platelets with APS absorbed with monocytes (Fig 4A) or with Glanzmann's platelets (Fig 4A, lane 2) confirms the IIb/IIIa nature of platelet lineage–specific antibodies in APS.

Studies on the lineage specificity of the IIb/IIIa complex were extended using purified glycoprotein IIb and IIIa to immunize rabbits. Of these two antisera only AIIIaS completely inhibited CFU-mix colony formation. This could have been due to low immunogenicity of platelet glycoprotein IIb, or alternatively it may indicate preferential expression of glycoprotein IIIa on the stem cell surface. Recent experiments with mouse platelet membranes separated by SDS-PAGE and transblotted onto nitrocellulose suggest that recognition of glycoprotein IIb (but not IIIa) by antibodies in APS is destroyed by SDS denaturation (P.J.A. McCaffery, personal communication), providing a basis for lower immunogenicity. Hence, the results confirm the presence of glycoprotein IIIa on the stem cell surface and provide evidence that IIIa bears a stem cell–megakaryocyte-platelet lineage-specific marker.

The existence of lineage-specific markers was proposed as early as 1976 by Till[27] who suggested that stem cells may express markers specific for each of the hematopoietic lineages within their capacity for differentiation. The existence of such lineage-specific antigen systems has been clearly demonstrated in the murine hematopoietic system,[28] and molecules expressing these antigens biochemically characterized in the T lymphocyte lineage,[29] and in the platelet lineage.[30] These studies have shown that the murine stem cell–megakaryocyte-platelet lineage marker is associated with the IIb/IIIa glycoprotein complex.[30]

Inherent within Till's model of a lineage-specific antigen system was the concept that such antigens might be involved in stem cell commitment and in the processes controlling differentiation along each particular lineage. In this regard we have briefly looked at a possible function of the IIb/IIIa complex on stem cells. The main role of this complex on platelets relates to adherence to endothelial cells by von Willebrand's factor or fibronectin and to aggregation with other platelets by fibrinogen bridging.[31,32] The demonstration that fibrinogen and fibrin fragment D have stimulatory effects on certain leukemic cell lines and on CFU-mix[8] suggests that the fibrinogen-binding domain of the IIb/IIIa complex might be expressed on the stem cell surface. In support of this contention we have demonstrated that platelet lineage–specific APS, which recognizes the stem cell, also inhibits fibrinogen-mediated platelet aggregation (Fig 5). However, in contrast, neither AIIIaS nor AIIbS (which also recognize the stem cell) inhibited platelet aggregation even at the highest antibody concentration attainable in this system. Again, this could be due to the low titer of both of these antisera. The observation that platelet numbers appear normal in Glanzmann's thrombasthenia argues against a role for glycoproteins IIb and IIIa in the control of differentiation along the platelet lineage unless the defect is limited to late stages of platelet-megakaryocyte maturation, or residual IIb/IIIa is sufficient for regulatory purposes at the level of the stem cell. In summary then, the function of glycoprotein IIIa on stem cells remains unclear though intriguing for its potential function in stem cell commitment and feedback regulation of the stem cell–megakaryocyte-platelet lineage. The next stage will be to raise monoclonal antibodies against cell lineage antigen specificities on the IIb/IIIa complex to have more precise probes for analysis of the stem cell–megakaryocyte-platelet lineage marker and its role in hematopoiesis.

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CONCISE REPORT

Clonable T Lymphocytes in T Cell–Depleted Bone Marrow Transplants Correlate With Development of Graft-*v*-Host Disease

By Nancy A. Kernan, Nancy H. Collins, Lisa Juliano, Teresa Cartagena, Bo Dupont, and Richard J. O'Reilly

Early clinical trials using T lymphocyte-depleted human marrow for transplantation have reported that such grafts reduce, to varying degrees, both the incidence and the severity of graft-*v*-host disease (GVHD). However, to date, no clear estimates have been made as to what degree of T cell depletion is necessary to prevent GVHD in every case. To address this problem, we used a limiting dilution assay (LDA) to quantitate residual clonable T lymphocytes in human T cell-depleted bone marrow in 31 HLA-identical transplants for leukemia. The number of phytohemagglutinin -interleukin 2–responsive T lymphocytes determined by LDA and expressed as T cell per kilogram recipient weight was found to correlate with the subsequent development of GVHD: no patients who received less than 1 × 10^5 T cell per kilogram developed GVHD (N = 24). Of the seven patients who received 1 × 10^5 to 4.4 × 10^5 T cell per kilogram, four patients developed grade I or II skin GVHD. This study thus provides a quantitative estimate of the number of T lymphocytes necessary to initiate clinically detectable GVHD in an HLA-identical host.

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GRAFT-*v*-HOST DISEASE (GVHD) following a bone marrow transplant (BMT) from an HLA-identical donor occurs in 50% to 70% of leukemic transplant recipients and is sufficiently severe (grade II through IV) to warrant treatment in 30% to 55% of cases, depending on the selection of pretransplant and posttransplant immunosuppression.[1,2] In several animal models it has been shown that removal of donor T lymphocytes from histoincompatible bone marrow[3-7] or spleens[8] can prevent GVHD. Conversely, addition of graded numbers of spleen cells or thymocytes to a marrow inoculum increases the incidence and severity of this reaction.[9,10] In one murine model of marrow transplantation, minor alloantigenic differences between an H-2–compatible donor and recipient result in a high incidence of lethal GVHD: contamination of 10^7 donor marrow cells with as few as 0.3% T cells (3 × 10^4 T lymphocytes) is sufficient to cause lethal GVHD.[11]

Preliminary clinical trials with bone marrow depleted of donor T lymphocytes indicate that both the incidence and the severity of GVHD in engrafted patients undergoing BMT for treatment of leukemia are greatly reduced.[12-16] However, neither the degree of T cell depletion required for the prevention of GVHD nor the number of T cells required to produce GVHD in humans has been established.

To quantitate the number of T lymphocytes in a bone marrow sample, we have developed a limiting dilution assay (LDA) that can detect 1 × 10^5 T lymphocytes in a total of 1 × 10^9 progenitor-enriched bone marrow cells.[17] In initial experiments, we demonstrated that a 3- to 4-log depletion of clonable T cells could be achieved by differential agglutination with soybean agglutinin (SBA), followed by depletion of cells forming rosettes with sheep red blood cells (E^-). We have now prospectively studied 31 BMTs that had been depleted of T lymphocytes (SBA^-E^-). The results of this analysis suggest that the number of residual clonable T lymphocytes correlates with the absence or occurrence of GVHD in leukemic patients who received HLA-identical T lymphocyte-depleted bone marrow.

MATERIALS AND METHODS

Thirty-one patients with leukemia received an HLA genotypically identical bone marrow that had been depleted of T lymphocytes. All patients were treated with protocols approved by the Internal Review Board at Memorial Sloan-Kettering Cancer Center, and informed consent was obtained before transplantation. The median age of the patients was 25 years (range, 1.5 to 42 years). The patients were prepared for transplant by cytoreduction with fractionated total body irradiation (13.2 to 14.4 Gy) and cyclophosphamide (120 mg/kg) as previously described.[18] All patients except one were hospitalized in single-room isolation without laminar flow or skin and mucosal decontamination. All patients received bone marrow that had been depleted of T lymphocytes by soybean lectin agglutinin (SBA) and E rosette depletion (E^-), according to a modification of the method of Reisner et al.[19] By the LDA used for the present report, this technique reproducibly achieves a 3- to 4-log depletion of clonable T cells,[17] while preserving hematopoietic progenitors.[19] None of these patients received any immunosuppression as prophylaxis against GVHD after BMT. Engraftment was documented by standard cytogenetic analysis, using either sex chromatin or quinacrine banding characteristics to differentiate donor and host hematopoietic cells. The diagnosis of GVHD was established according to the clinical criteria of Glucksberg et al[20] and the histological criteria of Slavin and Woodruff.[21]

Each T cell-depleted marrow was sampled before transplantation and evaluated for residual T lymphocytes by enumeration of E rosettes[22] and by a LDA technique that we have developed.[17] This

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technique determines the frequency of T lymphocytes responsive to phytohemagglutinin (PHA) and interleukin 2 (IL 2) in a sample of 1×10^6 bone marrow cells. This assay, described previously, has been modified to increase the T cell cloning efficiency. Briefly, T cell-depleted bone marrow fractions that had been maintained for up to 18 hours at 4 °C in medium-199 supplemented with DNAase 30 μ/mL (Sigma Chemical Co, St Louis), 10% fetal bovine serum, penicillin 100 μ/mL, and streptomycin 100 μg/mL were washed and resuspended with irradiated (4,000 rad) allogeneic peripheral blood mononuclear cells as feeder cells (0.5×10^6/mL) in RPMI-1640 (GIBCO, Grand Island, NY) supplemented with 15% pooled human serum, penicillin 100 μ/mL, streptomycin 100 μg/mL, L-glutamine 2 mmol/L/mL, PHA-16 4 μg/mL, and a prescreened optimum concentration of IL 2 containing supernatant derived from a Gibbon cell line (MLA), as previously described.[17] T cell-depleted marrow was seeded at 800, 600, 400, and 200 cells per well in 60-well Terasaki plates (Nunc, Thomas Scientific, Philadelphia). A total of 120 replicate samples per cell concentration were plated. After 11 days' incubation at 37 °C in an humidified atmosphere containing 5% CO_2 in air, the wells were scored as positive or negative for growth by microscopic examination. Because the relationship between the number of cells seeded per well and the fraction of negative wells at each concentration is a linear one, and thus consistent with single-hit kinetics, it is possible to establish an equation for the line of best fit using minimum chi-square statistics.[23] The slope of the line reflects the frequency of clonable T lymphocytes. The absolute number of T cells in a bone marrow inoculum was calculated by multiplying the frequency of T lymphocytes × the total number of mononuclear cells in the marrow inoculum. The use of DNAase in the maintenance medium and PHA-16 rather than PHA-P as the mitogen has increased the efficiency of the cloning of T cells from unmodified marrow to 75% to 80% of the number of E rosette-positive cells in the untreated bone marrow. In previous experiments, cytofluorographic analysis of cells pooled from positive wells from T cell-depleted BM demonstrated 70% to 95% expression of the pan T cell surface antigen CD3.[17]

The Kruskall-Wallis test was used to determine the statistical significance between the results obtained for each group of patients (GVHD *v* no GVHD).

RESULTS

Of the 31 patients analyzed, each achieved durable engraftment of donor progenitor cells with full reconstitution of hematopoietic function. Of these patients, two developed grade I skin GVHD (12.5 and 25 years) and two developed grade II skin GVHD (1.5 and 5 years). Twenty-seven patients did not develop GVHD, and no patient developed gastrointestinal or hepatic GVHD. In this series, there was no correlation between the age of the recipient or sex of the donor and the development or severity of GVHD.

The results of LDA quantitating residual clonable T lymphocytes demonstrated a significant difference in the T lymphocyte frequency ($P < .016$) and in the absolute number of T lymphocytes per kilogram ($P < .001$) between those patients who did and did not develop GVHD (Table 1). This difference is further demonstrated in Fig 1, in which the absolute number of residual clonable T lymphocytes determined by LDA and expressed as T cells per kilogram recipient body weight is plotted for each group of patients (panel A: no GVHD and panel B: GVHD). No patient who received less than 1×10^5 T cells per kilogram developed GVHD. Of the seven patients who received $>1 \times 10^5$ T cells per kilogram, four developed grade I or grade II skin GVHD. In the small sample of patients with GVHD, the dose of T cells (1.8 to 4.3×10^5 T cells per kilogram) did not correlate with the severity of GVHD (I or II).

In contrast, the development of grade I or grade II skin GVHD did not correlate with the number of residual T lymphocytes in the T cell-depleted graft as measured by the percentage of E rosettes. In addition, neither the absolute number of T cells nor the number of T cells per kilogram calculated by the percentage of E rosette-positive cells distinguished patients who did or did not develop GVHD.

DISCUSSION

Investigators in several laboratories have quantitated residual T cells in T cell-depleted marrow grafts by enumerating E rosettes or cells reactive with T cell-specific monoclonal antibodies, or by assaying for cells responsive to in vitro stimulation with mitogen or allogeneic cells in an attempt to identify transplants at risk for GVHD. While each of these analyses have documented significant reductions in the number of T cells transplanted after T cell depletion, they have not provided any consistent evidence of a correlation between the number of residual T cells transplanted and the subsequent development or severity of GVHD.[24] This may reflect the inability of these techniques to sensitively detect small numbers of T cells in large populations of actively proliferating marrow cells.

In our own initial analyses of a small series of SBA^-E^- marrow grafts, we were not able to identify residual T cells by E rosette analysis, reactivity with T cell-specific monoclonal antibodies, or response to mitogens or allogeneic

Table 1. Quantitation of Residual T Lymphocytes in GVHD

	No GVHD (N = 27)	GVHD (N = 4)	*P* Value
Limiting dilution analysis			
Frequency (10^{-3})	1.33 (0.16–5.5)	2.96 (1.84–7.4)	.016
T cells ($\times 10^6$)	1.93 (0.95–8.57)	6.78 (1.49–28.12)	.112
T cells per kilogram ($\times 10^4$)	3.72 (0.92–16)	23.99 (18.63–43.93)	.001
E rosettes			
Percentage	0.33 (0–1.5)	0.4 (0–3.7)	.856
T cells ($\times 10^6$)	4.00 (0–58.3)	1.22 (0–111)	.430
T cells per kilogram ($\times 10^4$)	6.31 (0–88.1)	30.04 (0–278.0)	.154

Results are expressed by the median and the range. *P* value was determined by the Kruskall-Wallis test.

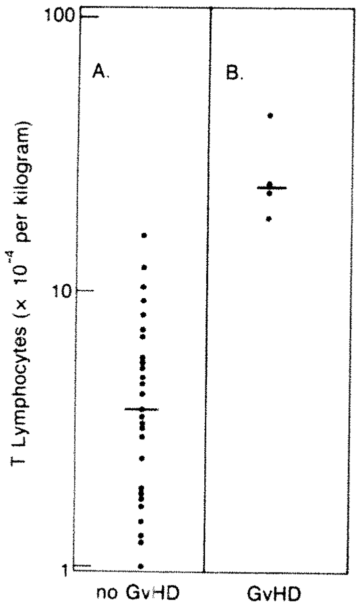

Fig 1. Absolute number of residual clonable T lymphocytes determined by LDA and expressed as T cells per kilogram recipient body weight.

cells.[19] Furthermore, T lymphocytes did not proliferate above background and could not be detected with T cell-specific monoclonal antibodies after the SBA^-E^- fraction was cultured in the presence of PHA and IL 2 for ten days.[17] However, residual T lymphocytes could be detected in the SBA^-E^- fraction, using conditions previously developed for T cell cloning. The latter technique provides optimal conditions for selective T cell growth from a small number of marrow cells. It has also permitted positive identification of growing T cells by monoclonal antibody analysis.

In the present study of 31 durably engrafted, HLA-identical SBA^-E^- T cell-depleted marrow transplants, we were also unable to show a correlation between the number of residual T lymphocytes detected in the final fraction by E rosette formation and the subsequent development of GVHD. However, a clear and significant correlation between the number of clonable T lymphocytes in the SBA^-E^- marrow inoculum, and particularly the dose of T lymphocytes per kilogram body weight, and the subsequent development of GVHD was observed. Patients receiving $<1 \times 10^5$ clonable T lymphocytes per kilogram did not develop GVHD, whereas four of seven patients receiving $>1 \times 10^5$ T lymphocytes developed this complication.

The disparity in results obtained by these two methods of T cell quantitation may be due to any of several possibilities. Detection of E rosettes or T cells reactive with monoclonal antibodies depends not only on the concentration of T lymphocytes, but also on the density of the antigens or receptors on the surface of these cells. Small populations of T cells expressing these determinants at low density might not be differentiable from non-T cells in marrow fractions at the time of the T cell depletion but could be detected if cultured under conditions that have been shown to promote T cell growth and T cell antigen expression. We also cannot rule out the possibility that populations of alloreactive postthymic T cells capable of initiating GVHD exist that do not express these T cell determinants, but can be induced to express them under these culture conditions.

Nevertheless, the frequency of clonable T lymphocytes in this LDA correlates with the development of GVHD, and the present study demonstrates for the first time a direct correlation between T lymphocytes in the bone marrow graft and subsequent development of GVHD.

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CONCISE REPORT

Absence of Tubular Structures and Immunolabeling for von Willebrand Factor in the Platelet α-Granules From Porcine von Willebrand Disease

By Elisabeth M. Cramer, Jacques P. Caen, Ludovic Drouet, and Janine Breton-Gorius

The electron microscopic localization of von Willebrand factor (vWF) was studied in platelets from normal and von Willebrand disease (vWD) pigs. In normal pig platelets, immunolabeling for vWF was far more intense and extensive than in human platelets and was either localized at one pole of the α-granule or all along its periphery or long axis. As in human platelets, this immunolabeling coincided with the presence of tubules about 200nm in diameter. These structures were more numerous than in human platelets, with up to 30 tubules per α-granule. They were easily identified either in transverse sections, usually grouped in a less electron-dense part of the matrix at one pole of the α-granule, or in longitudinal sections parallel to the long axis of the elongated granules, or coiled around the α-granule core. They closely resemble those structures found in Weibel-Palade bodies. In platelets from pigs with severe vWD, these structures were absent, as was the immunolabeling for vWF; however, cytoplasmic microtubules were normally present in these platelets. Thus, the granule-associated tubules can be distinguished from the microtubules, which are larger in diameter (250nm), are present in both normal and vWD platelets, and do not stain for vWF. These results strongly suggest that the tubular structures present in the α-granules of normal porcine platelets correspond to the vWF molecule itself.

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VON WILLEBRAND FACTOR (vWF) is present in platelets and plasma as a series of multimers ranging from 1 to 20 · 10^6 daltons.[1-3] This protein is secreted from platelet α-granules in response to various stimuli and is subsequently associated with the platelet surface.[4,5] vWF promotes platelet adhesion to subendothelium at the sites of vascular injury[6] under the conditions of high shear rates that prevail in small vessels.[7] In normal platelets, biochemical and immunological techniques have shown that vWF is distributed within the α-granules.[8-12] We have recently shown that in humans, the immunolocalization of vWF coincides with tubular structures[13] similar to those of Weibel Palade bodies, the vWF storage organelles of endothelial cells.[14,15] It was also recently shown that the processing steps in the biosynthesis of vWF by the megakaryocytes are similar to those of endothelial cells.[16] Von Willebrand disease (vWD) is defined by quantitative or qualitative abnormalities of vWF.[16] The virtual absence of the protein is the characteristic finding in severe vWD, an autosomal recessive disease that can affect both humans[17] and pigs.[18] In the present study, we have used electron microscopy to compare the immunolocalization of vWF and the ultrastructure of the tubules present in the α-granules of platelets from normal and vWD pigs in order to define the nature of the intragranular tubules. An abstract of this work was recently published.[19]

MATERIALS AND METHODS

Cells. Blood samples were taken from three normal control pigs (Large White colony) or from two pigs with vWD. The vWD pigs are from a colony maintained in the experimental farm of Institut National de la Recherche Agronomique (INRA, Jouy en Josas, France). The von Willebrand defect was introduced in the Large White colony by artificial insemination with semen from pigs affected with homozygous vWD (courtesy of Dr E.J.W. Bowie, Mayo Clinic, Rochester, Minn). Animals used in this experiment were from the fifth and sixth generations of cross-breeding of the first-generation offspring. The animals were considered as homozygous vWD pigs by the association of three criteria: (1) a bleeding time prolonged over 15 minutes by the ratio bleeding time[20]; (2) an undetectable level of vWF activity determined in the absence of ristocetin[21]; and (3) an undetectable level of vWF Ag determined by an enzyme-linked immunoassay (ELISA), using the same monospecific polyclonal antibody directed against porcine vWF as for immunolabeling.

Platelet isolation and assay of vWF Ag. Pig blood was collected in ACD (3.8 mmol/L citric acid, 6.2 mmol/L sodium citrate, 13.6 mmol/L dextrose, pH 6.5); platelet-rich plasma was obtained by centrifugation. The platelet pellet was resuspended in Tyrode's buffer (0.13 mol/L sodium chloride, 0.02 mol/L potassium chloride, 0.12 mol/L sodium carbonate, 0.005 mol/L glucose, pH 7.2) supplemented with 100 nmol/L prostaglandin E_1 and 1 mmol/L EDTA. The platelets were separated from plasma by filtration on Sepharose CL 2B. After counting by phase contrast microscopy, platelets were lysed with Triton X-100 and vWF Ag was determined by ELISA. The results were expressed in units ± 1 SD by comparison with the pool of normal pig plasma, assuming 1 mL of plasma contains one unit.

Standard electron microscopy and immunolabeling. Blood was drawn by venipuncture into plastic tubes containing 9 vol of fixative for each vol of blood according to Stenberg et al.[22] For standard electron microscopy, the fixative was 3% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Cells were fixed for one hour and washed three times in the phosphate buffer, postfixed in 1% osmium tetroxide, stained en bloc with uranyl acetate, and embedded in Epon. For immunocytochemistry, cells were prepared as previously described[13]: briefly, they were fixed in 1% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.2, for two hours at 22 °C, washed three times in the same buffer, and embedded in glycol-methacrylate.[23] vWF platelets were run in parallel with a control sample.

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The immunocytochemical reactions were performed on thin sections by the method of De Mey.[24] Sections were incubated for two hours in the rabbit antiporcine vWF serum. This antiserum was prepared by immunization of rabbits against purified porcine vWF and rendered monospecific by adsorption with vWD pig plasma as described for human vWD.[2]

Goat antirabbit immunoglobulin fraction coupled to 5-nm colloidal gold particles (GARG5) was purchased from Janssen Pharmaceutical (Beerse, Belgium) and used for one hour at a 1:10 dilution.

To demonstrate the specificity of the staining, control sections were incubated with nonimmune normal rabbit serum, followed by GARG5. One hundred platelets were examined in each sample and technical condition.

RESULTS

In platelets, the levels of vWF Ag were 14.4 ± 4.8 U/10^{10} platelets in normal pigs (n = 10) and 0.33 ± 0.18 U/10^{10} platelets in vWD pigs (n = 15).

Immunodetection of vWF in normal pig platelets. When porcine platelet sections were labeled with an anti-vWF antibody followed by immunogold, vWF was detected in the α-granules in a specific distribution pattern (Fig 1): gold particles were eccentrically located, labeling one pole of the α-granule. The surface of the α-granule was labeled either uniformly or all along its periphery and this pattern of labeling appeared to correspond to a tangential section of the α-granule. The labeling was intense, consistent, and reproducible and was only present within the α-granules of normal platelets. About 80% of the α-granules stained positively for vWF; the negativity of the others could be explained by the focal intragranular distribution of vWF. The cytoplasmic microtubules were not labeled, and similarly, the plasma membrane, mitrochondria, and the small granules, as well as the granules of other blood cells (neutrophils, monocytes, and lymphocytes), were not labeled. Control sections were consistently negative.

Standard electron microscopy of normal pig platelets. Porcine and human platelets could be distinguished by several morphological characteristics: porcine platelets were slightly smaller and the α-granules were generally uneven and larger (0.6 μm average diameter); the matrix of the porcine α-granules was cloudy and displayed an absence of nucleoid. The most striking difference, however, was the extent of the tubular structures previously shown to be colocalized with the immunolabeled vWF in human platelets[13] (Fig 2). When transversely sectioned, these tubules were either single or, more frequently, grouped, with up to 30 tubules per α-granule (Fig 3). Generally, the tubules were located in a special compartment of the α-granule which was of low electron density. Tubules were regularly spaced, and their average diameter was virtually identical to those in human platelets, ie, 200 nm. These structures could be distinguished from microtubules because of their slightly smaller diameter, the reduced thickness of their wall, the absence of a clear surrounding halo, and the occurrence of a central dense dot, possibly corresponding to a filament (Fig 4). In longitudinal sections, these tubules appeared to be regularly spaced, parallel, arranged along the long axis of the granules, and generally straight (Fig 5). Occasionally they exhibited a twisted course along the inside of the peripheral membrane of the α-granules (Fig 2).

Immunodetection of vWF in vWD pig platelets. When vWD platelets were immunolabeled for vWF, no specific labeling of any α-granule was observed (Fig 6). Furthermore, although occasional gold particles were observed in a few α-granules, they were not more concentrated than in the cytoplasm or other organelles, and this labeling was indistinguishable from the background staining.

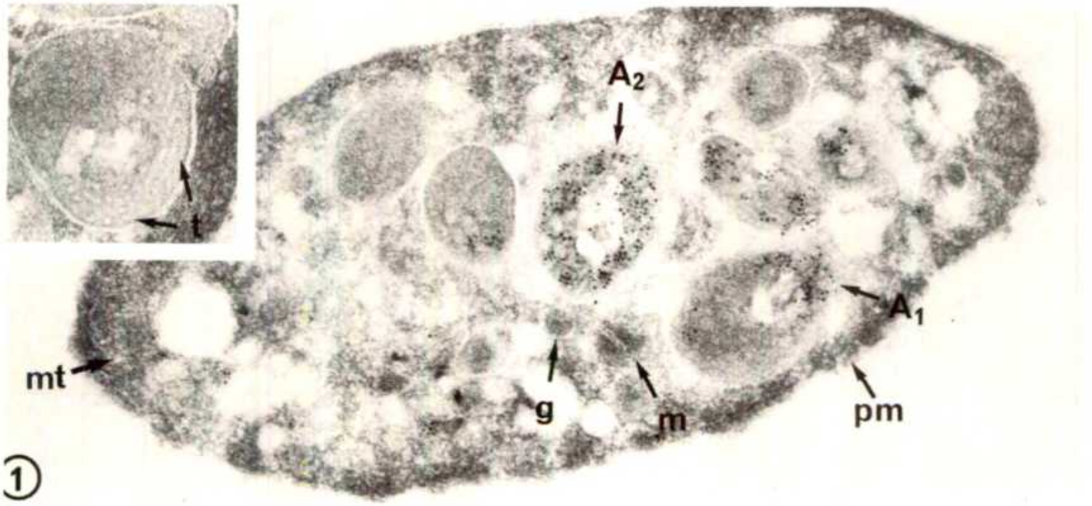

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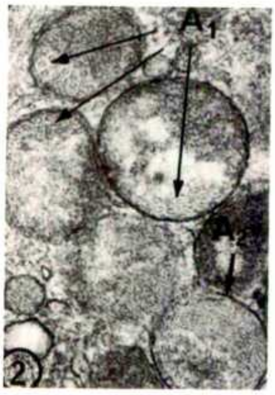
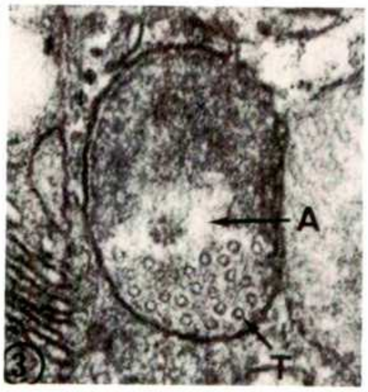
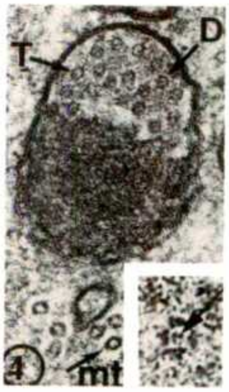
Fig 1. **Immunolocalization of vWF in normal pig platelets. The section of normal porcine platelets has been reacted with an anti-vWF antiserum followed by immunogold: the gold particles are numerous, labeling either one pole of the α-granules (A1) or the whole surface of some α-granules, possibly tangentially sectioned (A2). Cytoplasmic microtubules, mitochondria (m) smaller granules (g), and plasma membrane are not labeled. Original magnification ×50,700; current magnification ×43,095. (Inset) Control section of a typical α-granule, where the transverse and longitudinal sections of the tubules are observed. However, they are free of gold particles. Original magnification ×51,000; current magnification ×43,350.**







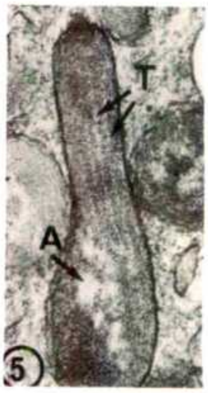


Fig 2. Normal pig platelets treated for standard electron microscopy. The α-granule displays many tubules either transversely (A1) or longitudinally sectioned and coiled along the periphery of the granule (A2). Original magnification ×40,800; current magnification ×34,680.

Fig 3. Normal pig platelets treated for standard electron microscopy. Higher magnification of an α-granule displaying at least 26 tubules (T) transversely sectioned. They are gathered in a compartment of the α-granule (A), where the matrix is consistently less electron-dense. Original magnification ×96,600; current magnification ×82,110.

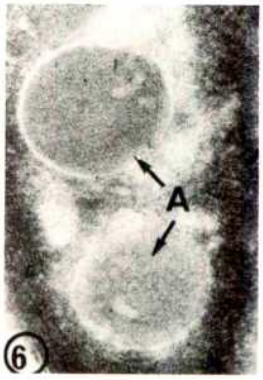
Fig 4. Normal pig platelets, showing different appearance of cytoplasmic microtubules (mt) and intragranular tubules (T). The microtubules display a characteristic clear halo, their wall is thicker, and their diameter slightly less than the other tubules. Occasionally the lumen of the intragranular tubules contains a dense central dot (D) visible in cross section. Original magnification ×92,100; current magnification ×78,285. (Inset) Higher magnification of the transverse section of a granule-associated tubule allowing a better visualization of the central structure (arrow). Original magnification ×235,000; current magnification ×199,750.

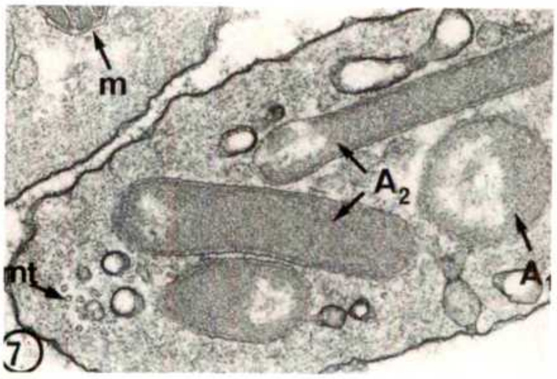
Fig 5. Longitudinal section of an elongated α-granule (A), displaying a parallel arrangement of the tubules (T) occupying the less electron-dense part of the α-granule and arranged parallel to its long axis. Original magnification ×50,700; current magnification ×43,095.

Standard electron microscopy of vWD pig platelets. The most striking feature of the ultrastructure of porcine vWD platelets was the absence of tubular structures within the α-granules (Figs 7 and 8). Indeed, in those areas of the α-granules where tubular structures would be expected, no distinctive structures could be observed. Despite absence of tubules, elongated α-granules were seen as often as in normal platelets and the cytoplasmic microtubules were present at normal levels.

DISCUSSION

We found that vWF from porcine platelets is localized in the α-granules as in human platelets,[8-13] but that the immunolabeling is more extensive than in humans. We have also shown that this extensive immunolabeling for vWF corresponds to the presence of numerous tubules that are located within a compartment of the α-granule. As in humans, these tubules have an average diameter of 200 nm[13] and are





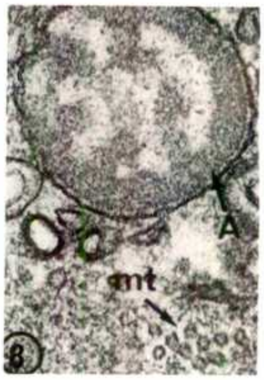


Fig 6. Section of a vWD pig platelet reacted for the vWF immunolocalization. No gold particles are observed on the α-granules (A). Original magnification ×52,000; current magnification ×44,200.

Fig 7. vWD platelets treated for standard electron microscopy. No tubular structures can be observed within the matrix of the α-granules (A1). Although some elongated granules (A2) are present, no longitudinal sections of tubules can be seen in their long axis. Cytoplasmic microtubules (mt) are present. Original mantification ×32,370; current magnification ×27,514.

Fig 8. Higher magnification of a vWD platelet showing the absence of tubular structures within the α-granules (A) and the presence of intracytoplasmic microtubules (mt). Original magnification ×95,000; current magnification ×80,750.

arranged in parallel to the long axis of elongated α-granules. These tubules closely resemble Weibel Palade bodies, both in size and because of the presence of a dense central dot, the latter possibly corresponding to a filament.[25] The similar eccentric localization of the immunodetected vWF and of these tubules suggests that they are colocalized and correspond to either parallel aggregates of vWF protein or vWF multimers[14] that are known to form long structures.[26] Alternatively, these tubules may be formed from structural proteins such as fibronectin[27] or other proteoglycans around which the vWF molecule could be coiled. However, their absence in vWD does not uphold this hypothesis. The quantitative results of the level of pig platelet vWF Ag obtained by ELISA cannot be compared with others since, to our knowledge, no such data are available in the literature.

The main finding of our study is the absence of the granule-associated tubules in parallel with the virtual absence of vWF from severe vWD platelets. We have compared the vWD porcine platelets with normal platelets and found that the immunolabeling of vWF is consistently negative in vWD platelets. However, although the level of vWF Ag measured by ELISA was extremely low in vWD platelets, it was not nil, suggesting that the immunolocalization technique has a limited sensitivity because only those antigens present at the surface of the section can be detected.[28] Thus, the absence of immunolabeling in this study does not exclude the possibility that traces of immunoreactive vWF would be below the threshold of sensitivity for this technique. In a study of long-term culture endothelial cells from vWD pigs, Booyse et al[29] occasionally found tubular structures in the cytoplasm that resembled authentic Weibel Palade bodies. In parallel, they also demonstrated that these cells displayed a slight fluorescence for vWF. In our study, both tubular structures and immunolabeling for vWF were absent. The differences between cell types and between in vitro and in vivo conditions may be an explanation of the discrepancies between the observations, but this remains to be demonstrated. Although no tubular structures were observed in vWD, several α-granules were elongated as in normal platelets, and therefore these tubules would appear to have no role in maintaining the elongated shape of the α-granules. The cytoplasmic microtubules were consistently present at the periphery of the vWD platelets and from their morphological appearance were clearly distinguishable from intragranular tubules.

In conclusion, this study suggests that the granule-associated tubules of normal platelets that are absent in vWD may represent the vWF molecule itself. It will be of great interest to extend this study in humans and to investigate platelet tubules in the different subtypes of vWD in which the nature of the defect may involve either antigen reactivity, degree of multimerization, or other abnormalities of vWF.[30-32] From such studies new information may arise on the nature of the intragranular tubules and their role in platelet function.

ACKNOWLEDGMENT

We are most grateful to Dr Dominique Meyer for preparing the porcine anti-vWF serum and for her contribution in the redaction of the manuscript, to Elisabeth Savariau for expert technical assistance, and to Dr John F. Martin and Dr Robert F.G. Booth for their kind editorial help.

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Autocrine Growth of Interleukin 2–Producing Leukemic Cells in a Patient With Adult T Cell Leukemia

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Leukemic cells in the peripheral blood of a patient with adult T cell leukemia (ATL), which expressed the Tac antigen/interleukin 2 (IL 2) receptor, were investigated in vitro for autocrine growth by IL 2. The cells showed spontaneous proliferation in mitogen-free medium. The spontaneous proliferation of the cells was inhibited by monoclonal anti-IL 2 or anti-Tac antibody. These cells were found to produce messenger RNA for IL 2 and secrete IL 2 during short-term culture in the same medium. Recombinant IL 2 and IL 2 secreted by the cells enhanced the proliferation of the cells in a dose-dependent manner when added to the initial culture. These findings demonstrate that an autocrine mechanism by IL 2 is involved in the proliferation of ATL cells during short-term culture.

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AN AUTOCRINE MECHANISM was demonstrated in the growth of malignant cell lines in vitro, and it was suggested that this mechanism could play a role in in vivo tumor growth.[1] Therefore, interleukin 2 (IL 2), an in vitro growth factor of activated T cell line,[2] is a possible candidate for an in vivo growth factor of some neoplastic T cells. The present study provides the evidence for the autocrine mechanism involved in IL 2, which was revealed in the leukemic cells of a patient with adult T cell leukemia (ATL). To our knowledge, the ATL cells of this patient demonstrate the first case of human leukemic cells that proliferate under this mechanism.

MATERIALS AND METHODS

Patient. A 55-year-old man from Kagoshima was admitted to our hospital with fever and leukocytosis (46,100/mm^3) with 62% abnormal lymphocytes possessing lobulated and convoluted nuclei. ATLA (ATL-associated) antibody in serum was positive (320 ×).

Characterization of the ATL cells. Peripheral blood was obtained from the patient, and mononuclear cells were separated by centrifugation on LSM (Ficoll-sodium diatrizoate) solution (Bionetics, Kensington, Md). One million cells were stained with monoclonal anti-Leu-1, -2a, -3a antibodies, anti-DR antibody, and anti-Tac antibody by indirect immunofluorescence, and fluorescein isothiocyanate (FITC)-conjugated goat antihuman immunoglobulin (Cappel) by direct immunofluorescence. The human T cell leukemia virus (HTLV) proviral genome was examined by the Southern blotting method with the use of a viral cDNA (U_3R)[3] as a probe (provided by Dr M. Maeda and Dr J. Yodoi of Kyoto [Japan] University).

Cell culture—IL 2 production and thymidine incorporation. The cells were suspended in RPMI-1640 (Flow Laboratories, Rockville, Md) medium containing 10% fetal calf serum (FCS, GIBCO, Grand Island, NY), 5×10^{-5} mol/L of 2-mercaptoethanol and antibiotics were placed at 2×10^6/mL in plastic culture dishes (Falcon 3002, Becton Dickinson, Oxnard, Calif) and cultured for varying time periods at 37 °C in 5% CO$_2$. The culture supernatant was harvested daily and IL 2 activity was assayed. The cells were also cultured at $2 \times 10^5/200\ \mu$L for varying time periods, using 96-well flat bottom tissue culture plates (Costar, Cambridge, Mass) and pulsed with 0.5 μCi per well of ^{3}H-thymidine (TdR) (6 Ci/mmol; New England Nuclear, Boston) for the last six hours, and the ^{3}H-TdR incorporation was measured after harvesting the cells.

Response of ATL cells to IL 2. The cells were cultured at $1 \times 10^5/200\mu$L with varying doses of IL 2 for 24 hours. The cultures were pulsed with 0.2 μCi of ^{3}H-TdR per well for the last six hours, and ^{3}H-TdR incorporation was measured. Dr J. Hamuro of Central Research Laboratories, Ajinomoto Co, kindly donated the human recombinant IL 2. The culture medium of ATL cells containing IL 2 activity was prepared by culturing the ATL cells at 5×10^6/mL in the medium for five days. The sample used contained 56.0 μ/mL of IL 2 activity.

Inhibition of cell proliferation by monoclonal anti-IL 2 and anti-Tac antibodies. The cells were cultured at $1 \times 10^5/200\ \mu$L with varying doses of anti-IL 2 (DMS2)[4] or anti-Tac antibody for 72 hours. The cultures were pulsed with 0.2 μCi of ^{3}H-TdR for the last six hours and then harvested.

IL 2 assay. The IL 2 assay was done using an IL 2-dependent mouse T cell line as described previously.[5] One tenth unit per milliliter of IL 2 gave 33% of the maximum response under the culture conditions in our study.

Detection of Il 2 mRNA by the Northern blotting analysis. IL 2 mRNA was detected as described previously.[6] Briefly, RNA was extracted from the ATL cells in the presence of guanidine isothiocyanate, and the glyoxalated RNA was electrophoresed through agarose gel and transferred to nitrocellulose as described. The RNA dot was hybridized with ^{32}P-labeled IL 2 cDNA (PstI-StuI fragment) as described previously. In control studies, tonsillar cells obtained from a patient with tonsillitis were used.

RESULTS

Characterization of the ATL cells. The cells expressed Leu-1 (96.3%), Leu-3a (86.0%), Tac (79.6%), and DR (69.9%) antigens but not Leu-2a antigen (1.2%) and surface

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immunoglobulin (1.0%). The cells had HTLV proviral genome.

IL 2 production and proliferation of the cells without mitogen. As shown in Fig 1, the ^{3}H-TdR incorporation of the cells was detected on the first day and reached a maximum on the third day. The IL 2 activity was detected in the medium on the second day and reached a maximum on the fifth day. Decrease of the TdR incorporation after the fifth day is probably due to depletion of the medium because the medium was not renewed during the culture period.

Detection of mRNA. The IL 2 mRNA was detected in the cells harvested on the fourth day of the culture (Fig 2). In control studies, a large amount of IL 2 mRNA was detected in tonsillar cells when stimulated with PHA-M and TPA, but not in tonsillar cells cultured in mitogen-free medium, indicating that normal lymphocytes do not produce detectable amounts of IL 2 mRNA when cultured without stimulation. These results indicate that these ATL cells are producing IL 2 without any mitogenic stimulation.

Response to IL 2. As shown in Fig 3, the proliferation of the cells was enhanced in a dose-dependent manner by both the recombinant IL 2 and IL 2 produced by the patient's own ATL cells. These results suggest that these ATL cells respond to IL 2 produced by themselves during culture.

Inhibition of proliferation by monoclonal anti-IL 2 and anti-Tac antibodies. The proliferation of the cells in mitogen-free medium was inhibited about 85% of the ^{3}H-TdR incorporation by both anti-IL 2 and anti-Tac antibodies but not by anti-DR antibody used as a control, as shown in Fig 4. These results definitively demonstrate that the in vitro growth of the ATL cells of our patient depends on their production of IL 2, and the proliferation is mediated through the interaction of the IL 2 receptors on the cells with IL 2 molecules.

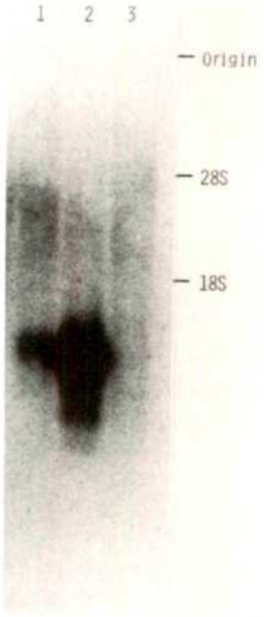

Fig 2. IL 2 mRNA is detected in the cells by Northern blotting analysis. RNA was extracted from the cells as described in Materials and Methods. The samples electrophoresed are lane 1, 40μg of RNA from the ATL cells cultured at 2 × 10^6/mL for four days in mitogen-free RPMI-1640–10% FCS medium; lane 2, 5 μg of Poly (A) + RNA from normal tonsillar cells cultured at 5 × 10^6/mL in RPMI-1640 medium for 16 hours with 1% phytohemagglutin (PHA-M: Difco Laboratories, Detroit) and 5 ng/mL of 12-*o* tetradecanoyl-phorbol-13 acetate (TPA: Sigma Chemical Co, St Louis) for 16 hours; lane 3, 40 μg of RNA from normal tonsillar lymphocytes cultured for 16 hours without PHA-M and TPA.

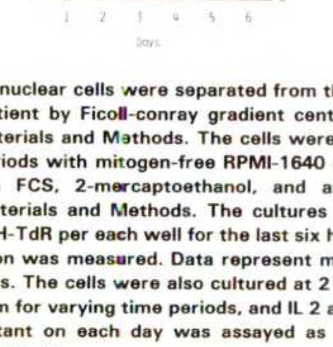

Fig 1. Mononuclear cells were separated from the peripheral blood of the patient by Ficoll-conray gradient centrifugation as described in Materials and Methods. The cells were cultured for varying time periods with mitogen-free RPMI-1640 medium supplemented with FCS, 2-mercaptoethanol, and antibiotics as described in Materials and Methods. The cultures were pulsed with 0.5 μCi of ^{3}H-TdR per each well for the last six hours and the TdR incorporation was measured. Data represent mean ± SD of triplicate cultures. The cells were also cultured at 2 × 10^6/mL in the same medium for varying time periods, and IL 2 activity in the culture supernatant on each day was assayed as described in Materials and Methods.

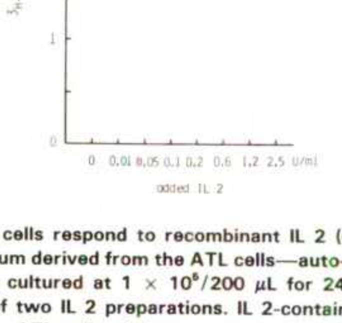

Fig 3. ATL cells respond to recombinant IL 2 (○) and IL 2-containing medium derived from the ATL cells—auto-IL 2 (●). The ATL cells were cultured at 1 × 10^5/200 μL for 24 hours with varying doses of two IL 2 preparations. IL 2-containing medium derived from the ATL cells of the same patient was prepared as described in Materials and Methods. The cultures were pulsed with 0.2 μCi of ^{3}H-TdR per each well for the last six hours and then harvested. Data represent means ± SD of triplicate cultures.

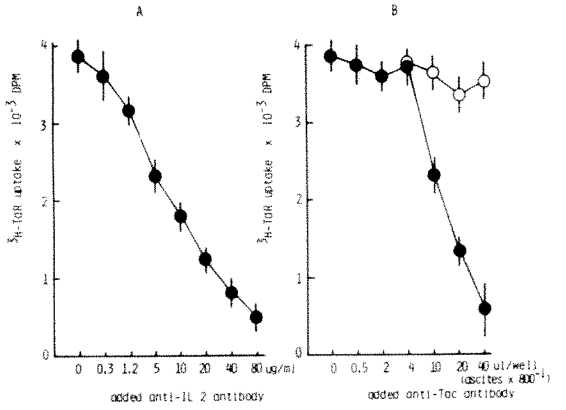

Fig 4. The proliferation of the ATL cells was inhibited by either anti-IL 2 and anti-Tac antibody but not by anti-DR antibody. The ATL cells were cultured at 1 × 10^5/200 μL in the medium described in Fig 1 with varying doses of anti-IL 2 (DMS2), anti-Tac, or anti-DR antibody for 72 hours. The cultures were pulsed with 0.2 μCi of ^{3}H-TdR per each well for the last six hours and then harvested. Monoclonal anti-IL 2 antibody used was purified by protein A (Bio-Rad, Richmond, Calif) affinity chromatography from the culture supernatant of DMS2 hybridoma. Anti-Tac and anti-DR antibodies used in this experiment were the ascites produced by hybridoma clones. Data represented means ± SD of triplicate cultures. (A) Effect of anti-IL 2 antibody (DMS2). (B) Effect of anti-Tac (●—●) and anti-DR (○—○) antibodies.

DISCUSSION

ATL is associated with HTLV.[7] However, in contrast to other RNA tumor viruses, HTLV lacks oncogene[8] and the integration sites of the HTLV in ATL cells are not common.[9] This indicates that the transcriptional activation of protooncogene of host cells by the insertion of viral genome at a common site, which appears to work in the case of avian leukosis virus,[10] seems to be unlikely in the case of ATL. On the other hand, it has been proposed from recent tissue culture studies with malignant cell lines that malignant cells respond to growth factors that they produce.[1] The possibility has also been raised that growth factors might be associated with the continuous growth of malignant cells in leukemia. In fact, Gordon et al[11] reported that the Epstein-Barr virus-transformed B cells respond to growth factor that they produce. Duprez et al[12] also recently reported that Tac-positive and RNA virus-negative human T cell line, IARC 301, proliferates by autostimulation, with IL 2 secreted by itself. As ATL cells also possess Tac antigen/IL 2R on the cell surface,[13] it is possible that these cells might continue their growth with the aid of an autostimulation mechanism by IL 2. Arya et al[14] and Yodoi et al[15] suggest that the continuous proliferation of ATL cells may result from IL 2-independent activation of the cells. However, our results support an autocrine mechanism, at least in our patient. It appears that this disagreement represents a heterogeneous leukemogenesis by HTLV, or the leukemogenesis follows the progression model proposed by Gordon et al[16] and Schrader[17] ie, initial utilization of a growth factor, followed by production of the growth factor in the next stage and the ultimate emancipation from the growth factor of tumor cell growth. In any case, a number of ATL patients should be studied before sweeping conclusions can be reached.

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CONCISE REPORT

Antithrombotic Effect of a Monoclonal Antibody to the Platelet Glycoprotein IIb/IIIa Receptor in an Experimental Animal Model

By Barry S. Coller, John D. Folts, Lesley E. Scudder, and Steven R. Smith

A murine monoclonal antibody directed at the platelet glycoprotein IIb/IIIa complex, which blocks platelet aggregation ex vivo, was tested for its antithrombotic effects in an established animal model of acute platelet thrombus formation in partially stenosed arteries. Infusion of 0.7 to 0.8 mg/kg of the F(ab')$_2$ fragment of the antibody completely blocked new thrombus formation despite multiple provocations, making it the most potent antithrombotic agent tested in this model.

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PLATELETS HAVE BEEN IMPLICATED in a variety of serious vaso-occlusive and thromboembolic disorders that together constitute the most common cause of death in the United States.[1] Although some studies with available antiplatelet agents have been encouraging, overall these drugs have shown only modest clinical efficacy, perhaps in part because none of them is capable of completely inhibiting platelet function at tolerable dosages.[1] We have recently prepared and characterized murine monoclonal antibodies to the platelet glycoprotein IIb/IIIa complex (GPIIb/IIIa) that, ex vivo, completely block platelet aggregation induced by the agonists thought to operate in vivo (adenosine diphosphate [ADP], epinephrine, thrombin, and collagen). They produce this inhibition by blocking the binding of fibrinogen (and perhaps von Willebrand factor, fibronectin, and thrombospondin) to the GPIIb/IIIa receptor.[2-4] More recently we showed that the infusion into dogs of F(ab')$_2$ fragments of one of these antibodies could produce nearly total inhibition of platelet aggregation measured ex vivo, and that this correlated with nearly quantitative blockade of the GPIIb/IIIa receptors.[5] The current study was designed to assess the antithrombotic effect of this antibody in a well-defined animal model of platelet thrombus formation in partially stenosed arteries.[6-8]

MATERIALS AND METHODS

The production of antibody 7E3, its radiolabeling with ^{125}I, and use in a platelet binding assay have been described in detail before.[2,3] For the present studies, antibody was purified from ascites by affinity chromatography on protein A-Sepharose, fragmented into F(ab')$_2$ with 2% pepsin, dialyzed against 0.15 mol/L NaCl, and frozen at −20 °C.[5] The absence of residual intact 7E3 antibody was confirmed by polyacrylamide gel electrophoresis. Since preliminary studies showed that the off rate of 7E3 F(ab')$_2$ fragments, like that of the intact antibody,[3] is very slow, it was possible to assess the number of F(ab')$_2$ fragments that bound to platelets in vivo. Thus, the difference in ex vivo binding of excess ^{125}I-7E3 to platelets obtained before and after infusion of the F(ab')$_2$ fragments represented the number of F(ab')$_2$ molecules bound in vivo.[5] Platelet aggregation in response to adenosine diphosphate (ADP) was also measured before and after infusion as previously reported.[2]

The cyclical blood flow reduction model has previously been described in detail.[6-8] Briefly, either the circumflex coronary artery of an anesthetized dog or the carotid artery of an anesthetized monkey is dissected out and an electromagnetic probe is placed on it to measure blood flow. The artery is stenosed approximately 70% by placing a 3- to 4-mm long encircling plastic cylinder of the appropriate internal diameter around the outside of the artery. Cyclical reductions in blood flow due to platelet thrombus formation then commence either spontaneously or after additional intimal damage is produced by brief (~1 second) clamping of the artery with a hemostat. Within several minutes, blood flow in the vessel decreases to a critical level, after which the flow is abruptly restored to its original level either by spontaneous embolization of the platelet thrombus or by embolization induced by gently shaking the cylinder. Once established, the cyclical flow reductions persist if no intervention is made. The frequency and amplitude of the flow reductions can be temporarily enhanced by infusing 0.5 µg/kg/min of epinephrine intravenously for ten minutes.[9]

The experimental protocol consisted of obtaining samples of blood anticoagulated with either 0.01 volume 40% sodium citrate (for platelet aggregation and ^{125}I-7E3 binding), or 0.037 volume 269 mmol/L EDTA (for platelet counts), and then performing the surgery. After cyclical flow reductions were established, another set of blood samples was obtained and the antibody (0.7 to 0.8 mg/kg) was infused intravenously as a bolus. A final set of samples was obtained 30 minutes after infusing the antibody. Platelet-rich plasma was prepared as previously described,[2] platelet counts on the platelet-rich plasma were performed by microscopy after dilution and lysis of erythrocytes (Unopette, Becton-Dickinson, Rutherford, NJ), and whole blood platelet counts were performed by an electronic resistive particle counter (S + IV, Coulter Electronics, Hialeah, Fla.). Unfortunately, in preliminary studies we were unable to obtain reproducible results with both the template and cuticle bleeding time methods; we are now trying to develop modified techniques for assessing this important hemostatic parameter in the experimental animals.

RESULTS

Cyclical reductions in blood flow were obtained in four dogs and four monkeys. In all four dogs and four monkeys,

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infusion of the F(ab′)$_2$ fragments resulted in complete cessation of the cyclical flow reductions and restoration of the control flow rate in ten minutes or less (Fig 1). In the dogs, restoration of coronary flow correlated with the disappearance of ST segment deviations on the electrocardiograms, indicating the reversal of myocardial ischemia. The cyclical flow reductions could not be restored with epinephrine infusions of 0.5 or 1 μg/kg/min for ten minutes, increasing the intimal damage by repeated brief clamping of the vessel with a hemostat, passing current (1 to 2 mA) through the cylinder, or a combination of these provocations (Fig 1). Platelet aggregation in response to ADP (≤25 μmol/L) was virtually abolished by the antibody infusion in all eight animals, although the shape change response remained intact (Fig 2). ^{125}I-7E3 antibody binding studies were performed on one dog and one monkey. In both animals 84% of the GPIIb/IIIa receptors were blocked by the F(ab′)$_2$ fragments (in the dog, 34,300 molecules of ^{125}I-7E3 bound per platelet before infusion and 5,500 after; in the monkey, 49,700 before and 8,100 after), a value nearly identical to

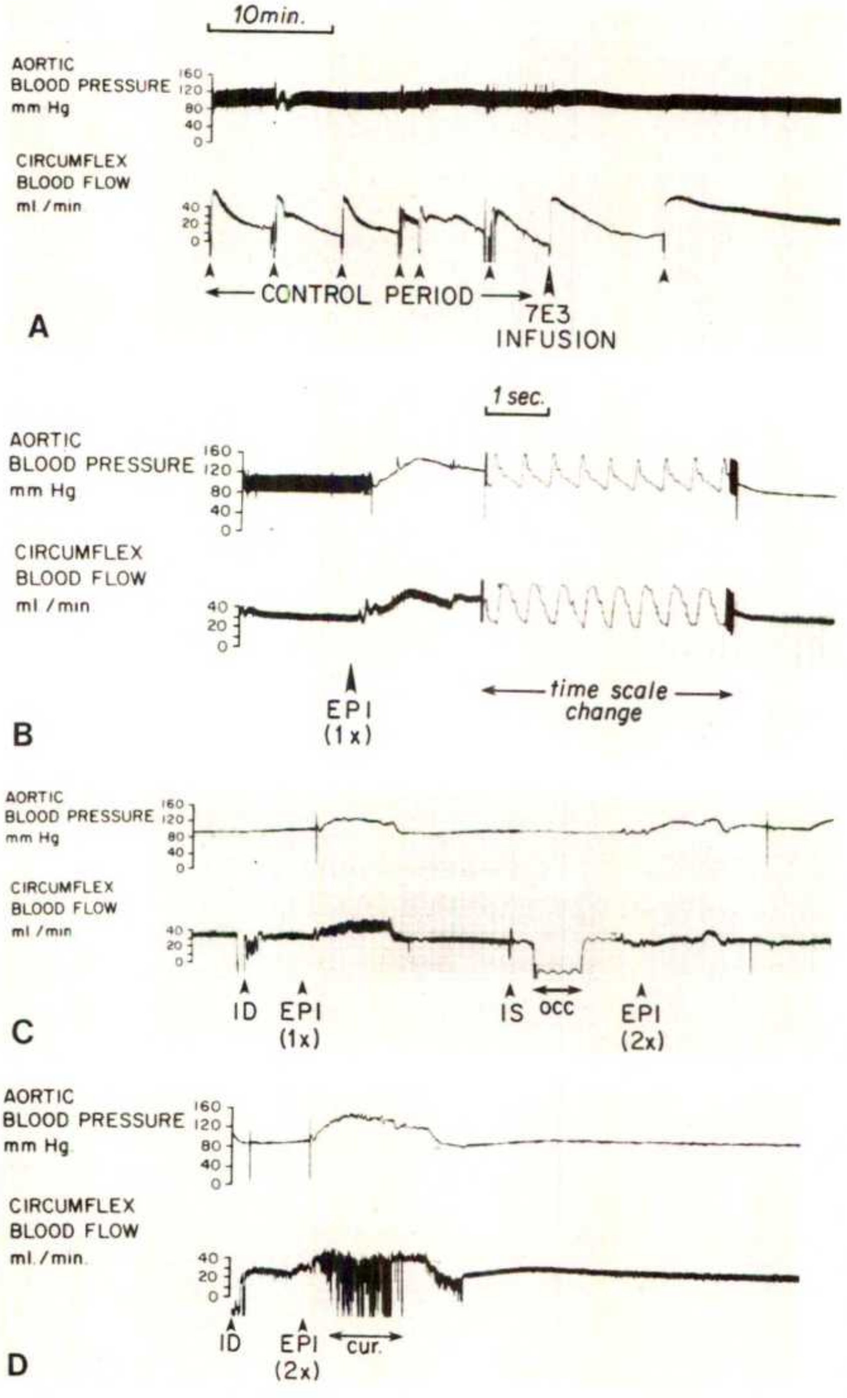

Fig 1. Continuous recording of arterial blood pressure (upper tracing; phasic in [A] and first part of [B] mean in last part of [B] and in [C] and [D]) and circumflex coronary artery blood flow (lower tracing) in a dog after partial stenosis of the circumflex artery. (A) Cycles of blood flow reduction, each of which was terminated by mechanical dislodgement of the thrombus (small arrowheads), were observed during the control period, after which the F(ab′)$_2$ fragment of 7E3 was injected (large arrowhead). The rate of decline in blood flow decreased almost immediately, and after a final dislodgement (small arrowhead) and a small decline in flow due to the decay of a mild hyperemic response, the increased flow was sustained. (B) The cycles were not restored with an epinephrine infusion (EPI 1X) (0.5 μg/kg/min for ten minutes), which actually increased the flow by increasing the blood pressure. (C) Increased intimal damage (ID) and a second epinephrine infusion (EPI 1X) also failed to restore the cycles. After slightly increasing the stenosis with a tapered string (IS) and demonstrating that this completely prevented a hyperemic response to total occlusion of the vessel (OCC), a double-dose epinephrine infusion (EPI 2X; 1 μg/kg/min for ten minutes) was given and it also failed to restore the cycles. (D) Finally, increased intimal damage (ID), a double-dose epinephrine infusion (EPI 2X) and passing current through the constricting cylinder (cur.) were combined, but still failed to restore the cycles.

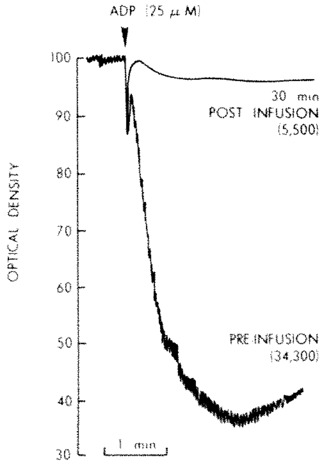

Fig 2. Inhibition of ADP-induced platelet aggregation by infusion of the $F(ab')_2$ fragment of 7E3. The platelets obtained before the infusion aggregated briskly in response to 25 μmol/L ADP, whereas the sample obtained 30 minutes after infusion showed an early increase in optical density reflecting shape change, but no aggregation. In the parentheses are the number of molecules of ^{125}I-7E3 that bound per platelet ex vivo, indicating that the $F(ab')_2$ fragments occupied 84% of the GPIIb/IIIa receptors.

that observed at this dose in the pilot study.[5] Platelet counts decreased by less than 15% in the three animals tested. None of the animals demonstrated a significant change in heart rate or blood pressure upon antibody infusion. There was no clear evidence of excessive hemorrhage from the operative sites after antibody infusion, although no objective data on blood loss were obtained.

DISCUSSION

These studies indicate that the $F(ab')_2$ fragment of antibody 7E3 is a potent antithrombotic agent in this model system. Considerable evidence accumulated by Folts et al[6-8] indicates that the reductions in blood flow in this model are due to interactions of platelets with the subendothelial surface in the area of stenosis, with increasing aggregation of platelets leading to vaso-occlusion.[6-8] The central role of platelet adhesion and aggregation in this model has been independently confirmed by several groups.[10-13] Although aspirin and other nonsteroidal antiinflammatory drugs inhibit the cyclical reductions in blood flow,[6-8] infusion of 0.5 μg/kg/min epinephrine for ten minutes consistently restores the blood flow reductions with all of these agents.[14-15] This augmentation of platelet thrombus formation by epinephrine may be important in understanding the effects of stress and cigarette smoking on cardiovascular disease, since both can elevate epinephrine levels.[16-18] Even prostacyclin does not completely abolish the cyclical reductions in flow after epinephrine infusion[9] and this is consistent with the ex vivo evidence that subaggregating doses of epinephrine can prevent inhibition of human platelet aggregation by prostacyclin.[19] Thus, since the inhibition of flow reductions by the $F(ab')_2$ fragment of antibody 7E3 was not reversed by epinephrine, it appears to be more potent than any of these antiplatelet agents. Only chlorpromazine has consistently protected against the augmented cyclical blood flow reductions produced by infusion of 0.5 μg/kg/min of epinephrine,[15] but preliminary evidence suggests that even this drug may not protect against cyclical reductions in blood flow produced by the 1 μg/kg/min dose of epinephrine also tested in this study. Moreover, the antibody fragment is considerably more potent than chlorpromazine in inhibiting platelet aggregation ex vivo.[15]

We chose to use $F(ab')_2$ fragments instead of intact antibody to avoid inducing thrombocytopenia or reticuloendothelial cell blockade in case the Fc portion of the murine monoclonal antibody is recognized by the animal's splenic macrophages. No significant reductions in platelet counts occurred during these acute experiments or with the more chronic experiments in the pilot study.[5]

Despite the profound inhibition of thrombus formation and ex vivo platelet aggregation, none of the animals developed spontaneous bleeding or clear evidence of excessive hemorrhage from their operative wounds. This paradoxical lack of dramatic bleeding despite total blockade of GPIIb/IIIa-mediated platelet aggregation measured ex vivo may be explained by the antibody leaving intact other platelet functions, including the interaction of von Willebrand factor with glycoprotein Ib, the interaction of collagen with another platelet receptor, and the ability to facilitate fibrin formation. Alternatively, the small number of residual unblocked GPIIb/IIIa receptors may have contributed to the animals' hemostatic integrity.

The efficacy of the monoclonal antibody fragment in this model can be ascribed to a fortuitous combination of factors, including the free access of the antibody to platelets in the circulation, the high affinity and specificity of the antibody for GPIIb/IIIa receptors, the absence of competing antigen in the plasma, the inability of platelets to undergo antigenic modulation, the maintenance of the platelet count, and the maintenance of sufficient qualitative platelet function to achieve hemostasis. The immunogenicity of murine monoclonal antibodies may, however, preclude their use as chronic therapeutic agents in humans, and there is no reason to believe that antiplatelet therapy would have a beneficial effect on established thrombi. Nonetheless, platelets have been implicated in the pathogenesis of a variety of ongoing acute phenomena, including preinfarction angina, transient ischemic attacks, acute occlusion of coronary arteries after thrombolysis or angioplasty, and acute occlusion of vascular grafts and anastomoses, that might potentially benefit from single-dose therapy with such antibodies. In fact, our preliminary data indicate that in a dog model, 7E3 $F(ab')_2$ fragments can prevent reocclusion of coronary arteries after thrombolysis with recombinant tissue plasminogen activator.[20] Additional studies will be required to establish whether such therapy would be safe in humans, and whether it has advantages over conventional antiplatelet agents.

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CONCISE REPORT

Thymic Regulation of Hematopoiesis III: Isolation of Helper and Suppressor Populations Using Counterflow Centrifugal Elutriation

By S.J. Sharkis, C. Cremo, M.I. Collector, S.J. Noga and A.D. Donnenberg

We have evidence that thymic regulatory cells can either enhance or inhibit the growth of hematopoietic progenitors in vitro. We have suggested that two separate populations are responsible for this regulatory interaction but isolation of the cell types has proven difficult. We now report the isolation by counterflow centrifugal elutriation (CCE) of two separate populations of thymocytes which regulate erythropoiesis in coculture. We demonstrate that a minority population (<10%) of slow sedimenting elutriated thymocytes provide a helper function whereas the suppressor population is the majority population. Furthermore, some thymocytes of intermediate cell volume neither enhance nor inhibit erythroid colony growth. We conclude that isolation of thymic subsets can lead to identification of populations which induce cell-cell regulation of hematopoietic progenitors resulting in both a positive and negative feedback control of growth.

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POPULATION(S) OF CELLS derived from the lymphohematopoietic organ systems have been implicated in the proliferation of erythroid colony forming cells.[1,2] In humans it was shown that in such disease states as Diamond-Blackfan syndrome[3] and aplastic anemia,[4] lymphocytes could inhibit erythroid colony formation in vitro. We have suggested, from work utilizing mixed cell populations in the mouse, that lymphoid cells from the thymus could regulate hematopoiesis both in vitro[5-7] and in vivo.[8] Whether the lymphoid cells act by direct cell-cell interaction (which results in a proliferation or antiproliferation event within the progenitor cells) or whether the regulatory populations release specific growth factors (i.e., interleukins) remains an area of active research and is open to further investigation. A major problem in dissecting these potential mechanisms has been an inability to physically separate the putative regulatory cell populations for further characterization. In order to determine if separate populations could be demonstrated which either enhance or inhibit erythroid colony formation, we sought to physically separate thymocyte populations using counterflow centrifugal elutriation (CCE), which can separate cells on the basis of size and, to a lesser extent, density.[9,10] We have determined that more than one population indeed could be demonstrated to regulate erythroid colony formation and have determined the relative distribution of cell types which could either enhance or inhibit colony formation.

MATERIALS AND METHODS

Animals. Male WBB6F$_1$ (WB/ReJ × C57Bl/6J) +/+ mice 5–7 weeks old were used as donors for both thymocytes and bone marrow cells.

Preparation of cell suspensions and coculture conditions. Mice were sacrificed by cervical dislocation, femurs and thymus removed and single cell suspensions prepared as described.[5] Coculture of constant numbers of bone marrow cells (5×10^4) with various (10^3 to 10^6) concentrations of thymocytes were prepared utilizing the plasma clot culture system for growth of erythroid colonies.[11] The thymocyte (1×10^9) were prepared as a single cell suspension in 5 to 10 mL of medium (0.9% saline, 0.5% bovine serum albumin [BSA], 0.1% d-glucose, 0.3 mmol/L EDTA at a pH 7.20) for processing by CCE.

Counterflow centrifugal elutriation. The use of predetermined flow rates at fixed rotor speed for the separation of leukocyte populations of small volume differences has been reported.[12,13] Briefly, the thymocytes were injected into the inlet stream of the Beckman J-6M centrifuge equipped with a JE-6B elutriator rotor and standard chamber (Beckman, Palo Alto, Calif). A master-flex peristaltic pump (Cole Palmer, Chicago, Ill) equipped with a potentiometer provides precisely metered flow. The cells were loaded into the chamber at a flow rate of 13 mL/min, rotor speed of 3,000 rpm and a temperature of 19 °C. A 50-mL eluate was collected and counted to confirm that fewer than 5×10^5 cells were eluted during loading. Rotor speed was held constant and the cells were eluted by changing flow rates. The flow rate was increased from 13 to 18 mL/min to collect the first fraction and cells collected by eluting to exhaustion (400 mL collection volume).

This process was repeated in stepwise fashion at 20, 24, and 28 mL/min. Cells remaining in the elutriator chamber designated the rotor-off (R/O) fraction were collected by continuing medium flow after stopping the rotor. Each fraction was concentrated by centrifugation (300 × g for 15 minutes), washed twice with RPMI 1640, counted, and cell volume distributions determined using a counter channelyzer (Coulter, Hialeah, FL). Cytospin preparations of thymocytes fractions were made and stained with Wright-Giesma. Thymocytes from each fraction were added to syngeneic donor marrow cells in coculture for the erythroid colony forming cell assay (CFU-e).

RESULTS

Separation of thymocytes by CCE resulted in cell populations of increasing cell volume as measured by the Coulter counter channelyzer (Fig 1). The increasing cell volume corresponded to cells collected at increasing flow rate. Thus, the fraction of cells collected at 13 to 18 mL/min had the smallest cell volume distribution and the R/O fraction the

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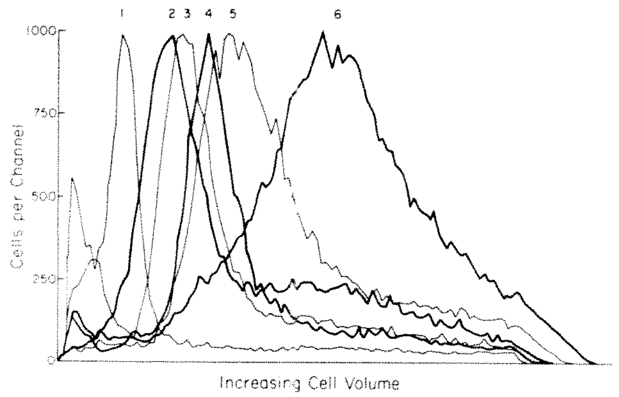

Fig 1. **Coulter counter channelyzer profile (a representative experiment) of the elutriated fractions of thymocytes. Numbers represent the cell volume distribution curves for the various fractions as follows: 1, 13- to 18-mL/min fraction; 2, 20-mL/min fraction; 3, 24-mL/min fraction; 4, unseparated thymocytes; 5, 28-mL/min fraction; and 6, the rotor-off (R/O) fraction.**

largest volume, respectively. The cytospin preparations confirmed this observation.

As can be seen in Table 1, the percent recovery of the various fractions (for 12 separate experiments) was markedly different, suggesting that there are several distinct populations of thymocytes of varying cell volume. The 13- to 18-mL fraction and the R/O fraction (the smallest and largest cell volume fractions) represent the minority recovered populations (3.5 ± 0.7% and 7.8 ± 0.9%, respectively) whereas the intermediate (24 mL/min) fraction represents the majority of thymocytes (48.1 ± 4.4%).

When these various fractions were cocultured with a constant number of marrow cells (5×10^4) for erythroid colony formation we found that the 13- to 18-mL/min fractions contained only helper cells, whereas the 24, 28, and R/O fractions contained the suppressor cells (Table 1). The 20- and 24-mL/min fractions contained varying numbers of cells which would neither help nor suppress (null) colony forming cell growth. The 20-mL/min fraction contained no suppressors and 10^6 of these cells gave the maximum enhancement. Thymocytes from each fraction cultured alone produced no colonies.

The data were analyzed by two-way analysis of variance for the effects of the number of thymocytes added to culture and the effect of the elutriation fraction from which the thymocytes were obtained. Both parameters were statistically significant ($P < 0.001$).

For the purpose of comparison, cloning efficiency in the cultures grown in the presence of 13- to 18-mL/min thymocytes or R/O thymocytes were contrasted with that of cultures grown in the absence of thymocytes. Cloning efficiency in cultures containing 13- to 18-mL/min thymocytes was significantly greater ($P = 0.003$) and efficiency in cultures containing R/O thymocytes was significantly lower ($P < 0.001$) than that of cultures grown without thymocytes.

Table 1. Addition of CCE-Enriched Thymocytes to Bone Marrow Cells for the Growth of Erythroid Progenitors (CFU-E)

Fraction (mL/min)	No. of Thymocytes Added*	% Total Cell Recovery (± SEM)†	CFU-E/5 × 10^4 Bone Marrow Cells Plated (± SEM)‡
—	—		155.4 ± 32.7
Unseparated	10^3		90.3 ± 30.4
	10^4	—	177.8 ± 44.3
	10^5		174.0 ± 35.0
	10^6		332.5 ± 54.8
13–18	10^3		243.9 ± 44.6
	10^4	3.5 ± 0.7	286.2 ± 47.6
	10^5		297.9 ± 50.6
	10^6		314.8 ± 29.3
20	10^3		160.9 ± 21.4
	10^4	28.4 ± 5.6	164.9 ± 31.0
	10^5		131.8 ± 40.8
	10^6		408.1 ± 50.9
24	10^3		97.5 ± 23.5
	10^4	48.1 ± 4.7	83.4 ± 22.6
	10^5		112.5 ± 23.9
	10^6		177.0 ± 35.9
28	10^3		94.4 ± 23.2
	10^4	12.1 ± 1.6	102.9 ± 21.2
	10^5		80.9 ± 18.5
	10^6		112.0 ± 41.4
R/O	10^3		97.5 ± 28.3
	10^4	7.8 ± 0.8	81.9 ± 31.2
	10^5		73.0 ± 19.2
	10^6		67.2 ± 23.0

CFU-E, erythroid colony-forming units. R/O, rotor-off fraction.

*Thymocytes from each fraction cultured alone produced no colonies.

†Cell counts of cells recovered in each fraction divided by total cells loaded (10^9 cells).

‡Number of colonies ± 1 SEM for twelve experiments.

DISCUSSION

We have proposed that enhancing and inhibiting functions could be attributed to separate populations of thymic regulatory cells for erythropoiesis.[5-7] The helper cell is in such small numbers in the thymus that finding, isolating, and purifying that population has proved difficult. Our previous hypothesis,[5] that 10^6 unseparated (20:1 thymus to marrow cell ratio in coculture) thymocytes were required to observe helper effects, took into account that this cell was a rare cell and a critical mass of these cells in coculture was required to provide enhancement of colony formation. Our current recovery data of the various fractions indicated the helper cell population was less than 10% of the total, a number predicted by earlier studies using the cell sorter to attempt to isolate these cells.[7] The suppressor cell population appeared to be the majority population in the 24-mL/min, 28-mL/min, and R/O fractions to varying degrees; however, it is the R/O fraction which contained only suppressor cells, whereas the 24- and the 28-mL/min fractions may contain some cells which are null.

The isolation of the rare helper cell in sufficient numbers will allow us to ask questions about the mechanisms by which it regulates (positively) the proliferation of stem cells. Furthermore, the suppression of colony formation can also be studied with purified regulatory cells from the R/O fraction. Can suppressors release one or more factors which inhibit erythroid colony forming cells? Several potential growth mediators come to mind. We have evidence[14] that polyamines which are present in large amounts in the thymus of rodents[15] may play a role in thymic regulation of erythropoiesis. Release of growth factors (ie, interleukins) by lymphoid cells could be important in the in vitro and in vivo proliferation of stem cells. We have some preliminary evidence that IL-3 can inhibit CFU-E. It is possible that lymphocyte types release different amounts of this growth factor, which in turn regulates colony formation. We are investigating this possibility. Our ability to isolate the rare helper cell and the more abundant suppressing cell in the thymus which positively and negatively regulate erythroid colony formation should now allow for clarification of the regulatory mechanisms involved in hematopoiesis.

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CONCISE REPORT

Stimulation of Proliferation of Human Myeloid Leukemia Cells in Culture: Applications for Cytogenetic Analysis

By Joseph Michaeli, Israela Lerer, Eliezer A. Rachmilewitz, and Eitan Fibach

The use of chromosome banding techniques has provided a valuable diagnostic tool in various malignancies. The application of these methods, however, is often restricted by a low yield of mitotic cells and the patient's unwillingness to comply with repeated bone marrow aspiration. In an attempt to promote mitotic activity of leukemic cells from the bone marrow and peripheral blood, we employed a new method based on culturing the cells in the presence of a conditioned medium derived from a human bladder carcinoma cell line (5637). In addition to colony stimulating factor, this conditioned medium contains a factor that is capable of stimulating leukemic myeloblast proliferation. Bone marrow and peripheral blood mononuclear cells from 58 patients with a variety of myeloid leukemias were cultured for 24 to 120 hours in the presence or absence of conditioned medium. These bone marrow cells showed a pronounced increase in the mitotic index (5- to 50-fold) as compared to unstimulated cultures, and a greater than 100-fold increase as compared to fresh, uncultured bone marrow cells. Analyzable metaphases could be obtained even in marrow samples in which direct or 24-hour G-banding techniques had failed to reveal metaphases. The effect observed on peripheral blood cells was even more dramatic because prior to culture no mitotic cells were detected, whereas up to 2% mitotic cells were found in conditioned medium–stimulated peripheral leukemic cells. Karyotype analysis of 36 out of the 58 leukemic patients has shown that the chromosome changes discovered in conditioned medium stimulated cells were identical to those found in unstimulated cells. New chromosome aberrations, attributable to the stimulation of growth by conditioned medium, were not found. The quality of the metaphases analyzed following conditioned medium stimulation was considerably better than that of unstimulated samples. Frozen cells, when cultured with conditioned medium, were also suitable for cytogenetic analysis. Thus, the use of this conditioned medium permits adequate cytogenetic analysis even in cases where such analysis was previously impossible.

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ALTHOUGH CYTOGENETIC STUDIES have become a valuable diagnostic tool for leukemic patients,[1] their use is often restricted by two major obstacles: the paucity or complete absence of dividing cells, rendering the bone marrow (BM) samples inadequate for analysis, and patient unwillingness to comply with repeated BM aspirations. Recently, we have demonstrated that a conditioned medium (CM) derived from cultures of a human urinary bladder carcinoma cell line (5637) is capable of stimulating the proliferation and growth of human myeloid leukemic cells (in preparation).

In an attempt to promote mitotic activity of leukemic cells and to permit cytogenetic analysis of peripheral mononuclear cells, we have employed a new method based on karyotype analysis of BM and peripheral blood (PB) leukemic cells cultured in the presence of this CM.

MATERIALS AND METHODS

Patients. The effect of the CM on the mitotic activity was determined in cultures of cells obtained from 58 patients with acute and chronic myeloid leukemia and the myelodysplastic syndrome. Informed consent was obtained from all patients. Detailed karyotype analysis was performed on 36 of these patients [15 with myelodysplastic syndrome, 11 acute myelogenous leukemia (AML) and 10 chronic myelogenous leukemia (CML)]. Cells from patients with myelodysplastic syndrome or acute leukemia were studied prior to initiation of chemotherapy. Three patients with acute lymphatic leukemia (ALL) and six normal controls were also studied. The type of leukemia or myelodysplastic syndrome was classified according to the French-American-British criteria,[2] following a review of Wright's stained PB smears, BM aspirates, and bone core biopsies. Cytochemical studies were also performed in all cases.

Cultures. Blood or marrow samples were collected in preservative-free heparin. In most cases they were processed immediately, and in other cases they were left overnight at room temperature before further processing. The mononuclear cell fraction was isolated using Ficoll-Hypaque density gradient centrifugation.[3] Cells from the interphase band were washed twice in phosphate buffered saline and cultured at 2.5 to 5 × 10^5 cells/mL in alpha minimal essential medium supplemented with 10% fetal calf serum (FCS) (GIBCO, Grand Island, NY) either in the presence or absence of 10% (vol/vol) CM. The cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C.

In some experiments human myeloid leukemic cells which had been stored were also studied. The cells were frozen as follows: PB and BM cells were isolated by Ficoll-Hypaque density gradient centrifugation, washed twice and resuspended at 2 to 4 × 10^7/mL in alpha medium supplemented with 10% FCS and 10% dimethylsulfoxide. One-mL aliquots were divided into 48 × 12.5-mm polypropylene tubes (Nunc, Rolkilde, Denmark) and frozen at 1 °C/min in a Linde CRFC-1 + CRC freezing units (Union Carbide, Indianapolis, Ind). Tubes were stored in liquid nitrogen. Before use, cells were thawed rapidly in a 37 °C water bath and washed twice in medium. Viable cells were assessed using trypan blue exclusion and cultured as described for fresh cells.

Determination of mitotic index. Since the percentage of mitotic cells in both fresh and cultured cells was usually <1%, we have utilized the following method for more accurate scoring. Approximately one million cells were treated with a hypotonic solution (0.075 mol/L KCl) for ten minutes and then fixed for ten minutes in a mixture of methanol:acetic acid (3:1). Following centrifugation,

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the cells were resuspended in approximately 100 μL of the same fixative. A 10-μL sample was diluted with water (1:10), mixed, and the concentration of cell nuclei was determined by hemocytometer. Additionally, 10 μL of the same sample was placed on a glass slide, dried, and stained with Giemsa. The mitotic cells were counted with a light microscope (×250 magnification). The percentage of mitotic cells (mitotic index) was calculated by dividing the number of mitotic cells on the slide by the total number of cells as calculated by counting on the hemocytometer. Using this procedure it was possible to score the mitotic cells in a population of 10^4 to 10^5 cells and to determine the number of mitotic cells even when at concentrations as low as 0.01%

Cytogenetic analysis. Samples were analyzed following 24 to 120 hours in culture in the presence or absence of CM. Samples cultured for 24 hours were synchronized with methotrexate.[4] Chromosome preparations were trypsin G-banded.[5]

Preparation of conditioned medium. The human bladder carcinoma cell line (5637)[6] was provided by Dr J. Fogh, Human Tumor Cell Line Bank, Sloan Kettering Institute for Cancer Research, Walker Laboratory, Rye, NY and was subcloned in our laboratory. One subclone was passed routinely every 2 weeks in alpha medium supplemented with 20% FCS. For production of CM, cells were seeded at $5 \times 10^5/75\ \text{cm}^2$ culture flask in 50 mL medium and serum. After 1 week of culture a confluent monolayer had developed; the supernatant medium was collected, centrifuged to remove cells and debris, filtered through a 0.2-μm Millipore filter and stored in aliquots at −20 °C.

RESULTS

The mitotic indices of PB and BM leukemic cells of representative cases prior to culture and after four days of culture in the presence or absence of CM of representative cases are shown in Table 1. Direct examination of PB cells from patients with either chronic or acute myeloid leukemia failed to reveal mitotic cells, even when patients had over $100 \times 10^9/\text{L}$ circulating blasts. The mitotic index of normal or leukemic fresh BM cells was <0.01%. Although mitotic cells were present in most unstimulated marrow leukemic samples, the mitotic yield was considerably higher (5- to 50-fold) in CM-stimulated BM cultures. Moreover, ample analyzable metaphases could be obtained in several marrow samples in which direct or 24-hour G-banding methodology had failed to reveal metaphases. Mitotic cells of CM-stimulated BM and PB samples were evident on day 1 and their number progressively increased during the following days (Fig 1). The rate of this increase, however, varied. In some patients it was evident as early as 24 hours following stimulation, whereas in others a longer period of culturing (48 to 120 hours) was required (Fig 2). When added to samples obtained from normal controls and patients with ALL, CM had no stimulatory effect during this culture period.

Table 1. Mitotic Index of Marrow and Peripheral Blood Cells Before and After Culture

		Mitotic Index (%)		
		Before	After Culture	
Diagnosis	Source	Culture	without CM	with 10% CM
CML	PB	<0.01	0.02	1.0
	BM	0.01	0.02	1.2
CML-BC	PB	<0.01	0.10	1.7
	BM	<0.01	0.08	0.9
AML	PB	<0.01	0.12	2.2
	BM	0.02	0.08	1.8
AMML	PB	<0.01	0.03	0.8
	BM	0.01	0.02	0.6
APL	PB	<0.01	0.01	0.3
	BM	<0.01	0.01	0.2
Normal	PB	<0.01	<0.01	<0.01
	BM	<0.01	0.05	0.10

Cells were cultured for 4 days with or without 10% 5637-CM.

The karyotype analysis of 36 CM-stimulated samples whether originated from PB or BM was virtually identical to the karyotype of the unstimulated samples. Nineteen patients exhibited one or more chromosomal abnormalities before stimulation. The same abnormalities were rediscovered in the CM-stimulated samples. One patient with myelodysplastic syndrome exhibited the following complex abnormalities: 44XX, −5; −7; −18; −20; 6P+ and two marker chromosomes. These abnormalities were also detected following stimulation. Fourteen patients had a normal karyotype both in CM-stimulated and unstimulated cells. In two patients with CML, the typical Philadelphia chromosome could be detected in PB cells only after CM stimulation, since no mitotic cells were found prior to culture. In one patient with AML, analysis of unstimulated samples revealed 18 mitotic cells with normal karyotype, whereas analysis following 24-hour stimulation with CM showed that 20% of the dividing cells exhibited an abnormal karyotype (40xy; 12P−).

CM effectively stimulated the growth of cells from freshly isolated samples, as well as cells derived from samples which were left overnight at room temperature. In addition, myeloid cells stored for 2 to 5 months in liquid nitrogen and thawed and cultured with CM were also suitable for cytogenetic analysis. Ten frozen samples were examined, three

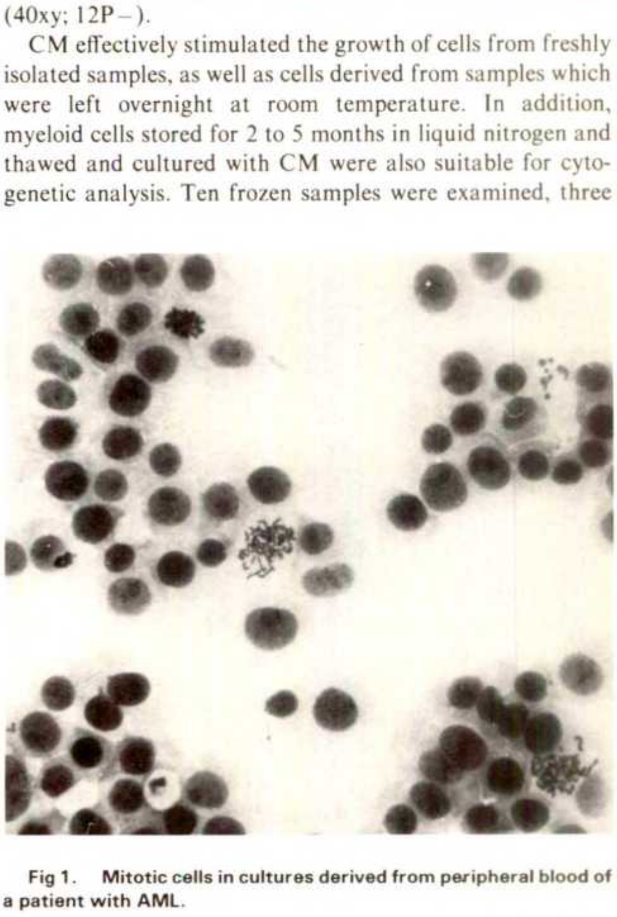

Fig 1. Mitotic cells in cultures derived from peripheral blood of a patient with AML.

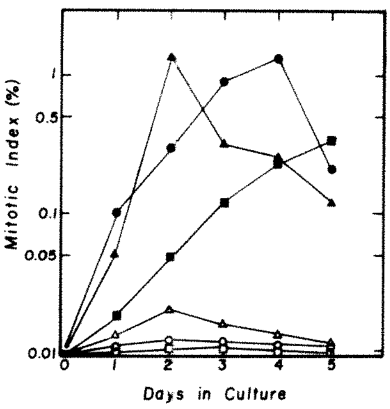

Fig 2. The mitotic index of peripheral blood myeloid leukemic cells in culture. Peripheral blood cells from three patients with AML were isolated on Ficoll-Hypaque and cultured either in the presence (full symbols) or absence (empty symbols) of 10% CM.

acute myelogenous leukemia (AML) (BM and PB cells), one acute promyelocytic leukemia (APL) (PB), one chronic myelogenous leukemia (CML) (BM and PB) and one CML in blast crisis (PB). The mitotic index of these cells was 0.1% to 1% within five days of culture, whereas the mitotic index of unstimulated cells was <0.01%. In patients with APL and CML, the characteristic translocations t(15:17) and t(9:22) were found even after the samples were frozen for an extended period (up to 5 months). The quality of metaphases found in both PB and BM samples was considerably better in CM-stimulated cells (Fig 3).

DISCUSSION

The recognition and identification of subtle chromosomal changes in leukemic cells has greatly been facilitated since the advent of high-resolution banding techniques.[4,5] However, efficient utilization of these methods is often hampered by the inadequate mitotic activity of the leukemic cells, and by the inconvenience of repeating marrow aspirations. An additional drawback stems from the uncertainty as to whether these methods detect chromosomal changes which are exclusively representative of the affected malignant clone.[7,8] In an attempt to circumvent some of these difficulties and to increase mitotic activity, we have performed cytogenetic analysis of human myeloid leukemic cells cultured in the presence of a CM which is derived from a human bladder carcinoma cell line (5637). A pronounced increase in the mitotic index of both PB and BM myeloid cells was found. Moreover, this culture technique substantially improved the ability to obtain analyzable metaphases even in samples in which direct or 24-hour G-banding methods had failed to reveal metaphases. This was relevant for BM, and particularly for PB cells, since their mitotic index prior to culture was consistently very low. The quality of metaphases in CM-stimulated samples, whether derived from BM or PB, was also improved (Fig 3). In addition, the ability to perform studies on frozen myeloblasts stimulated by CM was comparable to that of freshly isolated BM or PB cells. This method, therefore, may provide a useful aid for experiments where retrospective analysis of chromosomal changes might be needed.

Although we found that the CM was highly effective in enhancing cell proliferation, the rate of increase in the mitotic activity was not uniform. In some patients, maximal stimulation was noted shortly after incubation, whereas in others exposure for a longer period was necessary. It is advisable, therefore, that samples of CM-stimulated cells be evaluated repeatedly within 24 to 120 hours following incubation. The procedure described in this study for quantitative assessment of the mitotic index may therefore be valuable in determining the exact timing of analysis.

Although 5637-CM contains colony stimulating factors supporting the development of normal myeloid colonies in

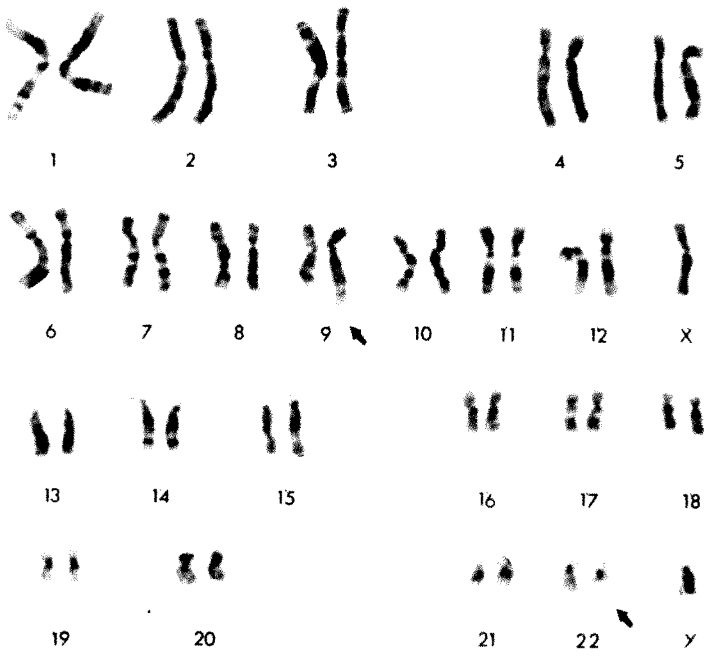

Fig 3. G-banded karyotype of a marrow sample from a patient with CML following 24-hour culture with 5637 CM. The karyotype is 46 xy t(9:22) (q34,q11). Arrows show t (9:22).

semisolid culture, it has only a negligible effect on the mitotic index of normal bone marrow cells in short term (1- to 5-day) liquid culture (Table 1). Rather it is likely that this CM contains a growth factor which is distinct from colony stimulating factor, and which specifically stimulates human myeloid leukemic cells (manuscript in preparation).

In addition to the unresponsiveness of normal cells to stimulation by CM in short term culture, we have shown that when samples which contained a chromosomal abnormality in all dividing cells were subsequently stimulated by CM, no enrichment of cytogenetically normal cells was found. It is therefore likely that the chromosomal analysis of CM-stimulated cells was confined to the malignant cell population.

Our study indicates that the chromosomal pattern of leukemic cells stimulated by CM for up to five days was consistent with that of the unstimulated cells. This was confirmed both when leukemic cells without detectable chromosomal abnormality and when cells exhibiting abnormal karyotypes were stimulated.

In one AML patient, however, a chromosomal aberration (46 xy; 12P−) could be detected only following stimulation with CM. In view of the fact that this deletion is known to occur nonrandomly in myeloid leukemias,[9] it seems that it represented an existing malignant clone that was exposed following stimulation by CM, rather than a new culture-induced alteration.

Although prolonged growth in culture may induce chromosomal changes, it would be of interest to culture for longer periods and to determine whether this may ultimately lead to the selection of new malignant clones. It would also be intriguing to explore the origin of such additional clones, whether they are merely culture artifacts or are truly representative of the patient karyotype. If the latter proves true, then the use of this CM for an extended duration may have prognostic value, as it may presage the clinical emergence of new malignant clones.

The determination of the percentage of cytogenetically normal cells in samples exhibiting mosaicism is also of prognostic importance, although this notion has recently been disputed in CML.[10] We are currently attempting to address this question by determining the proportions of cytogenetically normal and abnormal cells in CM-stimulated and unstimulated cells. Although in some patients we were able to detect more abnormal metaphases following stimulation, our data are still insufficient for final conclusions. We also intend to study whether the use of this CM or growth factor, by virtue of its specificity, will help detect minimal residual leukemic cells in patients attaining clinical remission.

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CORRESPONDENCE

EXPRESSION OF TARTRATE-RESISTANT ACID PHOSPHATASE

Letter to the Editor:

In their recent article on expression of tartrate-resistant acid phosphatase (TracP), Snipes et al[1] detected TracP activity in monocyte-derived macrophages cultured for three days, but not in the primary monocytes. These data confirm previous findings by Radzun et al[2] who observed TracP expression in "stimulated" monocytes, but not in normal peripheral blood monocytes.

What is puzzling in this article is the fact that induced U-937 cells remained TracP-negative. It is correct that untreated U-937 cells do not show the TracP band.[3] However, when incubated with the phorbolester TPA, U-937 cells clearly demonstrate TracP activity beginning on days 2 to 3 (depending on the concentration of the inducing agent) which increases over the following days as analysed by isoelectric focusing.[4] As U-937 cells show an overall "monocytic phenotype" and acquire macrophage features upon induction of differentiation, the expression of TracP in U-937 cells would rather support the conclusions drawn by the authors referring to the relationship between monocytic/phagocytic cells and bone osteoclasts.

Finally, we do not agree with the statement that, ". . . the latter technique [gel electrophoresis] is relatively cumbersome"; at least, the improved technique of isoelectric focusing on horizontal polyacrylamide thin-layer gels is fast and easy to perform and gives consistent and reproducible results. Furthermore, isoenzyme analysis appears to be more sensitive in detecting TracP expression than cytochemical staining.[5]

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We appreciate the comments of Dr Drexler regarding our recent publication in Blood.[1] One of our main goals was to determine whether monocytes, monocyte-derived macrophages, or U937 cells could produce the isoenzyme of acid phosphatase (Band 5b or E2) found in bone tissue. Radzun and coworkers[2] limited their investigation to the use of cytochemical staining. Our results suggest a good correlation between cytochemical staining and enzyme analysis. Regarding the use of gel electrophoresis, there is no doubt that it is more difficult to perform than cytochemical staining. However, we agree with Dr Drexler about the increased specificity of information from gel electrophoresis, as we emphasized in our article.

We were intrigued to learn that phorbol-treated U937 cells express a tartrate-resistant acid phosphatase band and we await publication of these results. Our work was limited to exposure of U937 cells to $1,25(OH)_2D_3$, which we have previously shown[3] causes differentiation of U937 cells and facilitates their ability to release radiolabeled calcium from bone chips.[4] The specific function of tartrate-resistant acid phosphatase is unknown, but it is not required for resorption of bone by phagocytic cells in vitro. Identification of conditions (eg, phorbol treatment) that allow U937 cells to express the tartrate-resistant acid phosphatase of bone further strengthens use of this cell line as a model for the study of osteoclast formation and function.[5]

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CONGENITAL METHEMOGLOBINEMIA AND MENTAL RETARDATION

To the Editor:

In a recent issue of Blood, Tanishima et al[1] reported two brothers with hereditary methemoglobinemia due to cytochrome b_5 reductase deficiency in erythrocytes as well as in lymphocytes, granulocytes, and platelets; the patients were not mentally retarded.

The coexistence of mental retardation with congenital methemoglobinemic cyanosis due to NADH-diaphorase deficiency does not seem to be a chance occurrence, but the relationship is not apparent. Fialkow et al[2] did not find decreased activity of NADH-diaphorase of the white blood cells in their diaphorase-deficient patients who were mentally retarded, and Tanishima et al[1] demonstrated this enzyme deficiency in all blood cells in the absence of mental retardation. In one of our families with mental retardation and congenital methemoglobinemia due to NADH-diaphorase deficiency, the two characteristics were not related.[3] I would suggest that NADH-diaphorase should be studied in all blood cells in most congenital methemoglobinemia cases prior to classification.

I would also like to point out that methemoglobin reductase deficiency is not always inherited as an autosomal recessive trait. Dominant inheritance could not be ruled out in one of our families,[4] or in Codounis'[5] family in which enzyme studies supported the genealogical findings.[6]

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To the Editor:

We acknowledge the letter of Dr. Özsoylu. The cause-and-effect relationship between mental retardation and hereditary methemoglobinemia due to NADH-diaphorase deficiency is not clear. However, the implication of this enzyme in lipid metabolism has been reported for cerebral tissues of animals.[1] The report of Junien et al[2] on two cases of fetuses at risk for generalized NADH-cytochrome b_5 reductase deficiency seems to support this view. Therefore, we suggest measuring the activity of NADH–cytochrome b_5 reductase rather than NADH diaphorase in all blood cells, and if possible in nonhematopoietic cells in congenital methemoglobinemia cases.

Many investigators now consider NADH–cytochrome b_5 reductase to be the major enzyme catalyzing the reduction of methemoglobin in vivo, and consider hereditary methemoglobinemia to be due to an abnormality in this enzyme.[3,4] Because NADH-diaphorase, measured by using dye as an electron acceptor of NADH, is not equal to NADH–cytochrome b_5 reductase, and it contains undefined NADH reductase, the measurement of NADH diaphorase might not be adequate for the detection of the enzyme deficiency. Furthermore, in platelets and leukocytes, the enzyme activity should be measured after careful extractions with detergents, as NADH–cytochrome b_5 reductase is a particle-bound enzyme and labile during storage. Some of the contradictory results reported previously should be reinvestigated according to the method developed recently for measuring NADH–cytochrome b_5 reductase.

As to the classification of type III enzyme deficiency, we cannot be certain. However, Panin et al[5] reported that several posttranslational events might determine the final degree of activity of the enzyme; some of these events might differ genetically in various types of cells, on the basis of the observation of changes in NADH–cytochrome b_5 reductase activity levels in erythrocytes and in leukocytes among the various age groups from newborns to adults.

Regarding the inheritance of methemoglobin reductase deficiency, Dr Özsoylu indicated that it was not always inherited as an autosomal recessive trait. He pointed out several cases of hereditary methemoglobinemia, including his own cases, which seemed to be inherited in an autosomal dominant manner. However, no enzymic data were given for methemoglobinemic and nonmethemoglobinemic patients except for selected individuals. An autosomal recessive trait seems to have been present in the families with the enzyme deficiency, judging from the results obtained by measuring NADH–cytochrome b_5 reductase activity.[6]

Thus, we would like to stress the importance of measuring NADH–cytochrome b_5 reductase activity, not NADH-diaphorase activity, for the detection and classification of hereditary methemoglobinemia due to the enzyme deficiency. Only after its careful determination in various tissues, together with biochemical studies on the role of the enzyme in nervous systems, can we better understand this interesting hereditary disease.

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while K562 ferritin has a high H-subunit content. It is important to point out that different subsets of noninduced HL-60 cells have ferritins differing in the ratio of H-subunits to L-subunits.[22] We found that noninduced HL-60 cells containing ferritin with a high H- to L-subunit ratio contained and released more acidic isoferritin inhibitory activity than noninduced HL-60 cells containing ferritin with a higher ratio of L- to H-subunits.[21] Furthermore, K562 cells produced a potent inhibitory activity not inactivated by heteroantibodies to acidic isoferritins.[3] Until this inhibitory activity is separated from ferritin, it is not possible to state whether or not K562 cells contain and release acidic isoferritin inhibitory activity.

Fourth, Sala et al[1] show in Table 7 of their paper that immunologically reactive ferritin can be found in medium conditioned for 18 hours by bone marrow cells. I assume they are trying to make the point that acidic isoferritins released endogenously should mask an effect of exogenously-added acidic isoferritins. Their studies are consistent with our own data demonstrating release of acidic isoferritin–inhibitory activity from monocytes into conditioned medium within 12 to 24 hours.[4,7,9] However, pulse exposure of bone marrow cells for an hour or less with acidic isoferritins results in maximal inhibition[4] and, within three to five hours of culturing cells at 37 °C, the cells become insensitive to inhibition by acidic isoferritins[4,11], unless they are reinduced into a state of responsiveness.[11] Thus, ferritin released by 12 to 18 hours would not interfere with suppression due to acidic isoferritins added exogenously at an earlier time.

In summary, I believe that the evidence still supports a role for acidic isoferritins as suppressor molecules of in vitro colony formation by normal myeloid progenitor cells.

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To the Editor:

Many of the points made by Dr Broxmeyer are discussed in our original paper[1] and we do not wish to repeat our earlier comments. The reason for carrying out this study was the difficulty in interpreting Dr Broxmeyer's data and his resulting thesis. A number of questions remain unanswered.

We are still unclear regarding his definition of an acidic isoferritin. In the original publication[2] this was defined in terms of isoelectric point and glycosylation. In later publications[3,4] it was held that the H subunit composition of the molecule was important in defining an acidic isoferritin. The reason for the failure of some H-rich isoferritins to inhibit colony formation was given as their nonglycosylated state and, in reply to an earlier criticism, it was stated[5] that the glycoprotein nature of the inhibitory activity was established. In the present letter Dr Broxmeyer reports that "purified recombinant, acidic H-subunit isoferritins" are inhibitory but the L-subunit molecule is not. Presumably these molecules are not glycosylated, which means that glycosylation is not required for suppressive activity. He also states that it is difficult to characterize acidic isoferritins only on the basis of the ratio of H to L subunits. We would therefore like to know how to identify an "acidic isoferritin" if isoelectric point, subunit composition, and glycosylation are not the defining characteristics.

We cannot comment on the purity of the ferritin preparations used by Dr Broxmeyer except to say that the "active" ferritin obtained from him and prepared from the spleen of a patient with chronic myelogenous leukemia (CML) contained very little ferritin.[1] That present was immunologically more similar to spleen ferritin ("L"-rich) than heart ferritin ("H"-rich). Purified and well-characterized isoferritins of various types prepared in this laboratory have been sent to Dr Broxmeyer and yielded entirely inconsistent results in his colony assay.

A further point of difficulty is the original claim that acidic isoferritins were active in suppressing colony growth at concentrations of 10^{-17} to 10^{-19} mol/L,[2] many orders of magnitude less than circulating concentrations in plasma and well below the limits of detection. In the paper by Dezza et al[6] cited by Dr Broxmeyer in support of his thesis, only one out of four pure ferritin preparations of H type inhibited colony formation at concentrations of 10^{-9} mol/L and 10^{-12} mol/L. This preparation showed only 5% binding to ConA. These authors note that the effective concentrations are higher than those found in human blood and question the physiological significance of the inhibition found. They also draw attention to the known ability of ferritin to bind smaller molecules[7,8] including lactoferrin, and speculate on the possibility that this could be responsible for some of the inhibitory effects.

We believe that our own results[1] and those of Dezza et al[6] make it difficult to sustain a clear picture of acidic isoferritins as regulators of hemopoiesis.

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ERRATUM

In reference to "Synchronized Cultures of *P falciparum* in Abnormal Red Cells: The Mechanism of the Inhibition of Growth in HbCC Cells" (Olson and Nagel, *Blood* 67(4):997–1001), many degenerating schizonts were detected in CC cultures on days 3 and 4, rather than on days 2 and 3 as stated in the Discussion section, page 1000.

ANNOUNCEMENTS

THE THIRD WILLIAM B. CASTLE SYMPOSIUM ON ADVANCES IN HEMATOLOGY

Friday, October 24, 1986
Grand Ballroom, Copley Plaza Hotel
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For further information, contact: Wilmot Cancer Research Fellowship Program, Medical Center Box 601, University of Rochester School of Medicine & Dentistry, 601 Elmwood Avenue, Rochester, New York USA 14642.

HOUSING ARRANGEMENTS FOR THE 1986 ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY

Multiple hotels will be utilized in San Francisco to house convention registrants for the annual meeting of the American Society of Hematology to be held Dec 6–9, 1986. Accommodations will be handled through the ASH Housing Bureau in San Francisco. To obtain your hotel reservation form, please write to the ASH Business Office, c/o SLACK Inc, 6900 Grove Rd, Thorofare, NJ 08086-9447.

THIRD INTERNATIONAL CONFERENCE ON MALIGNANT LYMPHOMA

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CALL FOR ABSTRACTS

Besides keynote lectures and invited presentations, there will be an opportunity to present other papers. Three poster sessions will be organized on the following topics: Hodgkin's disease, non-Hodgkin's lymphomas, and basic research in lymphoma. This conference will concentrate on lymphomas and immunodeficiency, cytogenetics, bone marrow transplantation, impact of molecular biology upon diagnosis and treatment of lymphoma, and new avenues for treatment of lymphomas.

Deadline for receiving abstracts is January 15, 1987. *For abstract forms and further information, contact:* F. Cavalli, MD, Head, Division of Oncology, Ospedale San Giovanni, 6500 Bellinzona, Switzerland; or telephone the conference secretariat at 091 / 23 62 10.

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NOTICE: AIDS Viruses

The International Committee on the Taxonomy of Viruses has approved a subcommittee's proposal to use the term "Human Immunodeficiency Virus (HIV)" for the retroviruses recently implicated as the cause of acquired immune deficiency syndromes (AIDS). This journal encourages prospective authors to use the recommended nomenclature as described in the May 9, 1986 issue of *Science* and the May 1, 1986 issue of *Nature*.

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Eligibility: Students, physicians in training, and doctoral or postdoctoral scientists who are in research training or were in research training as of June 1986. The applicant must be first author of the abstract and must plan to present the paper at the annual meeting of the societies in New Orleans in January 1987 if the abstract is chosen.

Procedure: Indicate that the abstract is to be considered for the Beecham Award on the Southern Meeting subspecialty classification sticker on the abstract submission form. A panel of judges will choose the finalists for the award from the abstracts submitted. Finalists will be asked to submit a manuscript to the President of the Southern Blood Club by December 1986.

Deadline for receipt of abstracts: September 30, 1986
Dates of the New Orleans Meetings: January 28–30, 1987

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SEMINARS IN DIAGNOSTIC PATHOLOGY	Quarterly
SEMINARS IN HEMATOLOGY	Quarterly
SEMINARS IN NEPHROLOGY	Quarterly
SEMINARS IN NUCLEAR MEDICINE	Quarterly
SEMINARS IN ONCOLOGY	Quarterly
SEMINARS IN ONCOLOGY NURSING	Quarterly
SEMINARS IN OPHTHALMOLOGY	Quarterly
SEMINARS IN ORTHOPAEDICS	Quarterly
SEMINARS IN PERINATOLOGY	Quarterly
SEMINARS IN RESPIRATORY INFECTIONS	Quarterly
SEMINARS IN ROENTGENOLOGY	Quarterly
SEMINARS IN ULTRASOUND, CT, AND MR	Quarterly
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GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

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**The Cohn-Oncley process used to produce GAMIMUNE® N has been challenged in vitro with a virus spike of 1×10^5 of HTLV-III and found to remove and/or inactivate the viral challenge.[1] In addition, GAMIMUNE® N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 reduction of HTLV-III.[1] In a separate study conducted by the Food and Drug Administration,[2] an in vitro challenge at each of six fraction steps using HTLV-III demonstrated a cumulative reduction of greater than 1×10^{15} infectious units.*

Please see following page for brief summary of prescribing information.

NEW NATIVE IGIV

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease, averages 40% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~0.3 mEq/g protein), a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

U.S. License No. 8

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Hereditary hemochromatosis is a common autosomal recessive disorder that results in body iron overload and damage of liver, heart, joints, endocrine and other organs. This symposium will consider basic science and clinical problems concerned with hemochromatosis in humans. The topics to be covered include: genetics, regulation of intestinal transfer of iron and delivery to cells, pathogenesis of cellular iron toxicity, clinical features, effects of therapy and the screening of families and the general population for hemochromatosis.

There will be contributed poster sessions. The deadline for submission of abstracts is November 15, 1986. A 200-word summary of work to be presented should be sent to: Dr. Corwin Edwards, Associate Professor of Medicine, University of Utah College of Medicine, Rm. 4B-120, 50 North Medical Drive, Salt Lake City, UT 84132.

Conference chairs:

Corwin Q. Edwards, MD.—University of Utah College of Medicine
Margaret A. Krikker, M.D.—The Hemochromatosis Research Foundation, Inc.
Lewis R. Weintraub, M.D.—Boston University School of Medicine

Inquiries should be addressed to: Conference Department, The New York Academy of Sciences, 2 East 63rd Street, New York, New York, 10021.

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Please see brief summary
of prescribing information
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LEUCOVORIN CALCIUM

LEUCOVORIN CALCIUM INJECTION
Military Depot: NSN 6505-01-054-7008,
50 mg/vial for I.M. use

DESCRIPTION: Each 1 ml ampul of Leucovorin Calcium *Lederle* contains Leucovorin 3 mg as the calcium salt which is the form preferred for intramuscular injection. Preservative: benzyl alcohol 0.9% w/v. The inactive ingredients are sodium chloride 0.56% w/v, Water for Injection qs 100% and sodium hydroxide or hydrochloric acid is used to adjust the pH to approximately 7.7.

Each 50 mg vial of Leucovorin Calcium cryodesiccated powder when reconstituted with 5 ml of sterile diluent contains Leucovorin 10 mg per ml as the calcium salt which is the form preferred for intramuscular injection. Contains no preservative. Dilute only with Bacteriostatic Water for Injection USP which contains benzyl alcohol. The inactive ingredients are sodium chloride 40 mg/vial, and sodium hydroxide or hydrochloric acid qs to pH approximately 8.1. When reconstituted as directed, the resulting solution must be used within seven days. If the product is reconstituted with Water for Injection USP use immediately.

ACTIONS: Leucovorin (folinic acid) is the formyl derivative and active form of folic acid.

Leucovorin Calcium is useful clinically in circumventing the action of folate reductase. There is no evidence that intramuscular doses of greater than 1 mg have greater efficacy than those of 1 mg.

INDICATIONS: Indicated (a) to diminish the toxicity and counteract the effect of inadvertently administered overdosages of folic acid antagonists. (See Warnings). (b) In the treatment of the megaloblastic anemias due to sprue, nutritional deficiency, pregnancy, and infancy when oral therapy is not feasible.

CONTRAINDICATIONS: Not to be administered for the treatment of pernicious anemia or other megaloblastic anemias where Vitamin B_{12} is deficient.

WARNINGS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to lack of Vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

In the treatment of overdosage of folic acid antagonists, Leucovorin must be administered within one hour, if possible, and is usually ineffective if administered after a delay of four hours.

PRECAUTIONS: In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive.

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

DOSAGE: Megaloblastic anemia: No more than or up to 1 mg daily. There is no evidence that intramuscular doses greater than 1 mg daily have greater efficacy than those of 1 mg; additionally loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg.

For the treatment of overdosage of folic acid antagonists: To be given in amounts equal to the weight of the antagonist given.

HOW SUPPLIED:

NDC 0205-4004-51 – 6 – 1 ml Ampuls
NDC 0205-5330-92 – 50 mg Vial of Cryodesiccated powder

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Rev 11/80

LEUCOVORIN CALCIUM TABLETS

BRIEF SUMMARY

Before prescribing Leucovorin Calcium Tablets, please consult complete prescribing information.

INDICATIONS AND USAGE: Leucovorin Calcium Tablets are indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (eg, methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS: *General:* Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia, a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin, and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with leucovorin. It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Leucovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother.

Pediatric Use: (See Drug Interactions.)

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for 72 hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the premethotrexate serum creatinine, the leucovorin dosage should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below 5×10^{-8}M.[7,8] The recommended dosage of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (ie, trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[9,10,11]

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Rev 2/86

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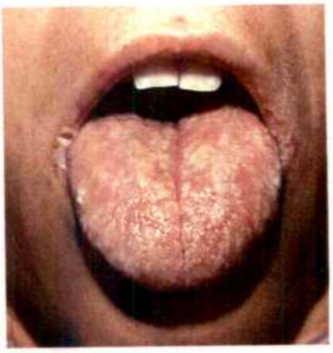
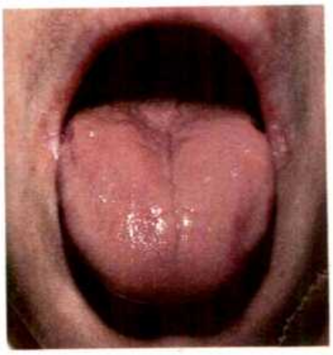
The first troche treatment for oropharyngeal candidiasis.


Before treatment.[1]


After treatment with MYCELEX Troche.[1]

High clinical cure* rates.

A clinical cure rate of 96% was achieved in one randomized double-blind trial[2] in which MYCELEX Troche was administered to patients with oropharyngeal candidiasis. Other investigators have concluded that MYCELEX Troche "is highly effective treatment for chronic oral candidiasis."[3]

Dramatic improvement in symptoms.

Patients attain a high degree of relief at the end of therapy, as evidenced by the dramatic decrease in severity of glossitis, patches, perleche, pain, and other clinical signs and symptoms of thrush.[4]

Good patient acceptance, minimal side effects.

With MYCELEX Troche, messy suspensions and lengthy swishing are things of the past. MYCELEX Troche has a pleasant, slightly sweet taste. And an entire day's therapy—5 troches—can easily be carried in pocket or purse.

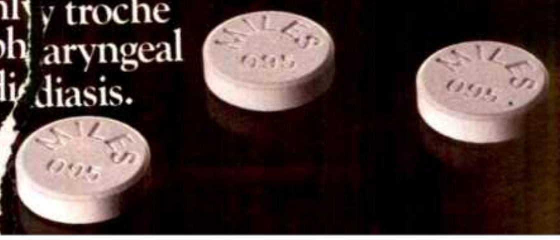
Based on a study of 52 episodes of oropharyngeal candidiasis, the investigators stated: "We believe that the troche form of clotrimazole...in the treatment of thrush in any patient population...is both effective and safe."[2†]

*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

Mycelex® Troche

(clotrimazole) 10 mg



MYCELEX® (clotrimazole) TROCHE

Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C: Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals.

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For further information contact:

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[1] Rubin, L. A., C. C. Kurman, M. E. Fritz, W. E. Biddison, B. Boutin, R. Yarchoan, and D. L. Nelson. 1985. Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. **The Journal of Immunology** 135:3172.

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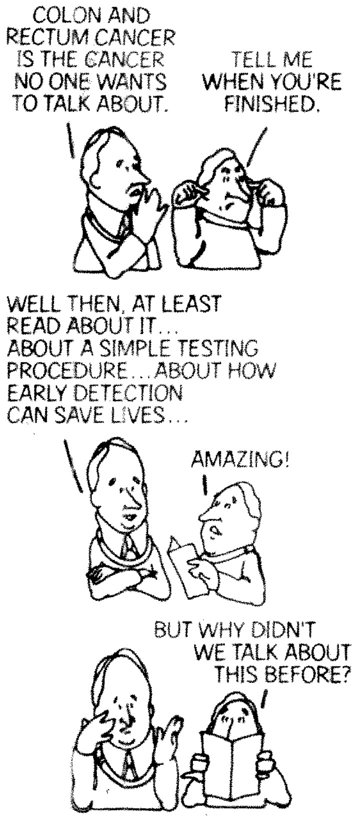
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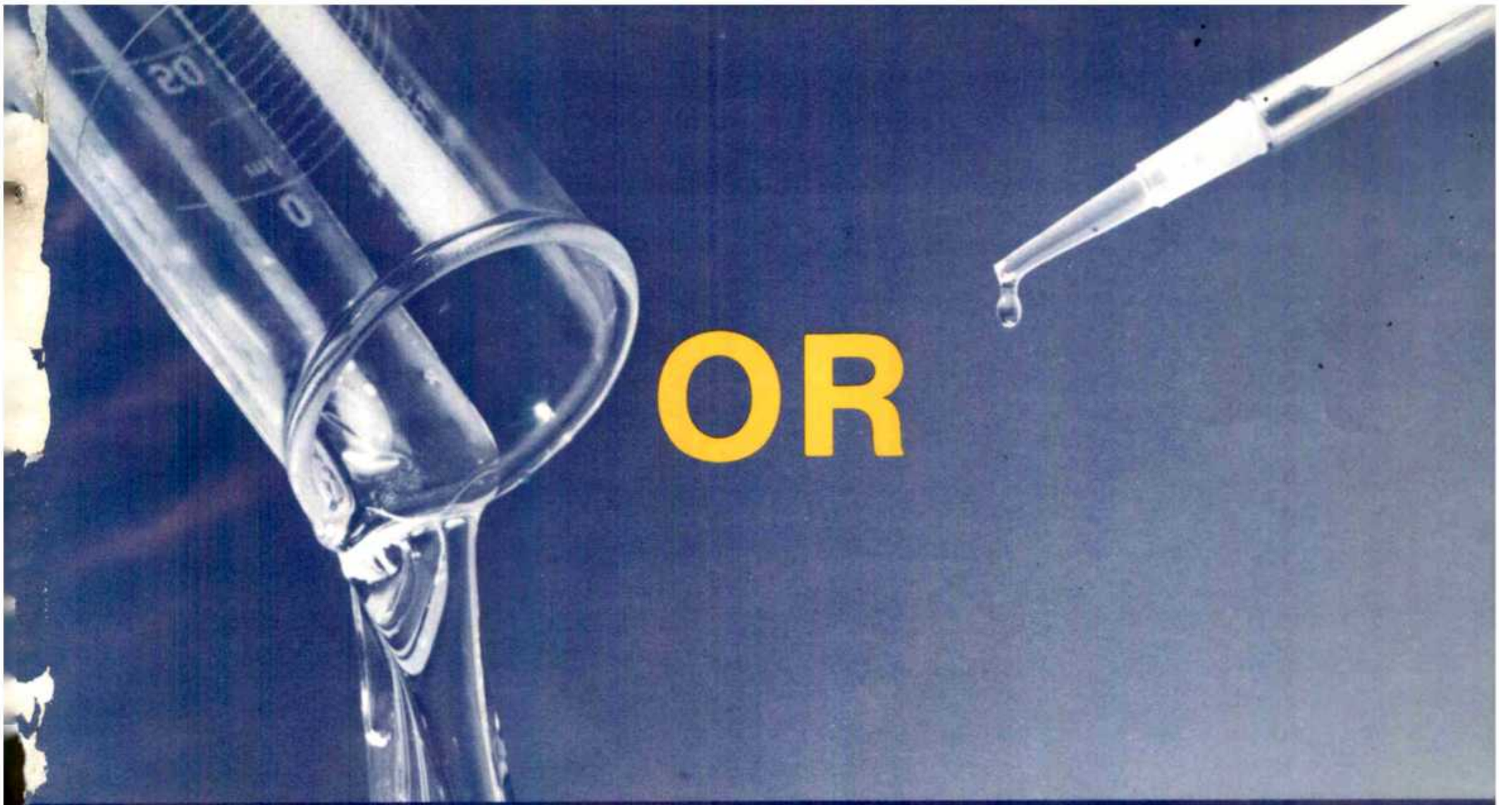

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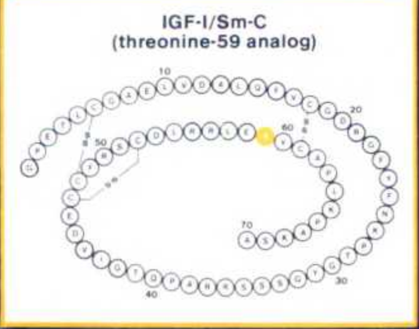
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